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(71)(72) Applicant and Inventor: MURGITA, Robert, A. [US/CA]; 17 Parkside Place, Montreal, H3H 1A7 (CA).

(74) Agent: CLARK, Paul, T.; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110-2804 (US).

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(54) Title: RECOMBINANT HUMAN ALPHA-FETOPROTEIN AND USES THEREOF

(57) Abstract

In general, disclosed are methods of inhibiting autoreactive immune cell proliferation in a mammal, involving administering to the mammal a therapeutically effective amount of recombinant human alpha-fetoprotein or an immune cell anti-proliferative fragment or analog thereof; methods of inhibiting a neoplasm in a mammal, involving administering to the mammal a therapeutically effective amount of recombinant human alpha-fetoprotein or an anti-neoplasm fragment or analog thereof; and methods of cell culture, involving the use of a media containing recombinant human alpha-fetoprotein or a fragment or analog thereof.

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RECOMBINANT HUMAN ALPHA-FETOPROTEIN AND USES THEREOF

Background of the Invention

This invention relates to the expression and purification of cloned human alpha-fetoprotein; methods for treating autoimmune diseases; cancer therapeutics and diagnostic methods; and cell growth and cell culture.

Alpha-fetoprotein (AFP) is a serum protein normally found at significant levels only in fetal blood. In adult blood increased alpha-fetoprotein levels are associated with liver regeneration and certain carcinomas.

Summary of the Invention

In general, the invention features substantially pure biologically-active recombinant human alpha-fetoprotein including a sequence that is substantially identical to either amino acids 1 to 389 of Fig. 1 (SEQ ID NO: 9) or a fragment thereof; amino acids 198 to 590 of Fig. 1 (SEQ ID NO: 10) or a fragment thereof; amino acids 198 to 389 of Fig. 1 (SEQ ID NO: 7) or a fragment thereof; amino acids 390 to 590 of Fig. 1 (SEQ ID NO: 8) or a fragment thereof; and amino acids 266 to 590 of Fig. 1 (SEQ ID NO: 11) or a fragment thereof.

In another related aspect, the invention features a method for using an insect cell for producing biologically active recombinant human alpha-fetoprotein or a fragment or analog thereof involving

a) providing a transformed insect cell (e.g., Spodoptera frugiperda) including a recombinant DNA molecule encoding the human alpha-fetoprotein or fragment or analog thereof operably linked to an expression control element which directs the expression of the human alpha-fetoprotein or fragment or analog thereof;

b) culturing the transformed cell; and

c) recovering the biologically active human alpha-f toprotein r fragment or analog there f.

In related aspects, the invention also features substantially pure human alpha-fetoprotein or a fragment or analog thereof produced using any of the methods described herein, and therapeutic compositions including substantially pure human alpha-fetoprotein (or a fragment or analog thereof) produced using any of the expression systems described herein.

In another aspect, the invention features a method of inhibiting autoreactive immune cell proliferation in a mammal (e.g., a human patient), involving administering to the mammal a therapeutically effective amount of recombinant human alpha-fetoprotein or an immune cell anti-proliferative fragment or analog thereof. Such a method is based on my discovery that unglycosylated recombinant human alpha-fetoprotein which is made in a prokaryote (e.g., E. coli) is useful for inhibiting autoreactive immune cells derived from a mammal. Preferably, such immune cells include T cells or B cells; and the recombinant human alpha-fetoprotein (or an immune cell anti-proliferative fragment or analog thereof) used in such methods is produced in a prokaryotic cell (e.g., E. coli) and is unglycosylated.

In another aspect, the invention features a method of treating an autoimmune disease in a mammal (e.g., a human patient), involving administering to the mammal a therapeutically effective amount of recombinant human alpha-fetoprotein or an immune cell anti-proliferative fragment or analog thereof. Such an autoimmune disease is multiple sclerosis; is rheumatoid arthritis; is myasthenia gravis; is insulin-dependent diabetes mellitus; or is systemic lupus erythematosus. In other preferred embodiments the autoimmune disease is acquired immune deficiency syndrome or involves a rejection of a

transplanted organ, tissue, or cell. Preferably, the recombinant human alpha-fetoprotein used in such methods is produced in a prokaryotic cell (e.g., E. coli) and is unglycosylated. In other preferred embodiments, such methods further involve administering to the mammal an immunosuppressive agent in an effective dose which is lower than the standard dose when the immunosuppressive agent is used by itself. Preferably, such an immunosuppressive agent is cyclosporine; is a steroid; is azathioprine; is FK-506; or is 15-deoxyspergualin. In yet another preferred embodiment, such a method involves administering to the mammal a tolerizing agent. Preferably, the recombinant human alpha-fetoprotein used in such methods is produced in a prokaryotic cell (e.g., E. coli) and is unglycosylated.

According to the invention, administration of recombinant human alpha-fetoprotein ("rHuAFP") (or a fragment or analog thereof) can be an effective means of preventing or treating or ameliorating autoimmune diseases in a mammal. To illustrate this, I have shown that recombinant HuAFP produced in a prokaryotic expression system is effective in suppressing T cell proliferation in response to self antigens, despite the fact that such rHuAFP is not modified in the same fashion as naturally occurring HuAFP. The use of natural HuAFP has heretofore been limited by its unavailability, natural HuAFP is obtained by laborious purification from limited supplies of umbilical cords and umbilical cord serum. Because biologically active rHuAFP can now be prepared in large quantities using the techniques of recombinant DNA, the use of rHuAFP for treating autoimmune diseases is now possible. The use of rHuAFP is especially advantageous since there are no known adverse side effects related to human alpha-fetoprotein

and it is beli ved that relatively high doses can be saf ly administ red.

In still other aspects, the invention features compositions and methods for the protection, treatment, and diagnosis of neoplasia, in particular, cancer. This aspect of the invention is based on my discovery that unglycosylated recombinant human alpha-fetoprotein made in a prokaryote (e.g., E. coli) is useful for treating and diagnosing mammals with neoplasms, especially malignant tumors, such as breast or prostate carcinomas, and other carcinomas caused by a proliferation of malignant cells which express receptors which are recognized by recombinant human alpha-fetoprotein.

In one aspect, the invention features a method of inhibiting a neoplasm in a mammal (e.g., a human patient), involving administering to the mammal a therapeutically effective amount of recombinant human alpha-fetoprotein or an anti-neoplasm fragment or analog thereof. Preferably, the neoplasm is a malignant tumor (e.g., a breast tumor or a prostate tumor); and the recombinant human alpha-fetoprotein is produced in a prokaryotic cell (e.g., E. coli) and is unglycosylated. In preferred embodiments, the cells of the neoplasm express a receptor which is recognized by the recombinant human alpha-fetoprotein. Such a neoplasm is generally a carcinoma such as an adenocarcinoma or a sarcoma. In preferred embodiments, the neoplasm proliferates in response to a hormone, e.g, estrogen or androgen. Preferably, administration of recombinant human alpha-fetoprotein inhibits the proliferation of cells of the neoplasm or, alternatively, kills cells of the neoplasm in the mammal. The method further includes administering to the mammal a chemotherapeutic agent.

In another aspect, the invention features a method of protecting a mammal from developing a neoplasm,

involving administ ring to the mammal a therapeutically ffective amount of recombinant human alpha-fet protein. Preferably, the recombinant human alpha-fetoprotein is produced in a prokaryotic cell (e.g., E. coli) and is unglycosylated.

In another aspect, the invention features a hybrid cytotoxin including a recombinant human alpha-fetoprotein (or a fragment or analog thereof) linked to a cytotoxic agent. Examples of such cytotoxic agents include, without limitation, diphtheria toxin, Pseudomonas exotoxin A; ricin and other plant toxins such as abrin, modeccin, volkensin, viscumin; chlorea toxin (produced by Vibrio cholerae bacteria); the so-called "Shiga-like" toxins (produced by E. coli and other enteric bacteria); Salmonella heat-labile enterotoxin; and E. coli heat-labile enterotoxin. In other preferred embodiments, the cytotoxic agent is non-proteinaceous. Examples of such non-proteinaceous cytotoxic agents include, without limitation, anti-cancer agents such as doxorubicin, as well as α-emitting radionuclides such as astatine and β-emitting nuclides such as yttrium. Preferably, the cytotoxic agent of the hybrid cytotoxin is linked by a peptide bond to the recombinant human alpha-fetoprotein, and the hybrid toxin is produced by expression of a genetically engineered hybrid DNA molecule. In other preferred embodiments, the cytotoxic agent of the hybrid cytotoxin is a protein; such a cytotoxic agent is chemically conjugated to the recombinant human alpha-fetoprotein.

In other aspects, the invention features a detectably-labelled recombinant human alpha-fetoprotein or a detectably-labelled fragment or analog thereof capable of binding to a human neoplastic cell. Preferably such a molecule is labelled with a radionuclide, e.g., technetium-99m, iodine-125, iodine-

131, or indium. Oth r detectabl labels includ , without limitati n, enzymes, fluorophor s, or other moieties or compounds which emit a detectable signal (e.g., radioactivity, fluorescence, color) or emit a detectable signal after exposure of the label to its substrate or, alternatively, the detectable signal can be an epitope recognized by an antibody (e.g., an epitope of alpha-fetoprotein or an epitope which is specifically engineered into the recombinant alpha-fetoprotein such as the HA or myc epitopes). Preferably, the molecule targets a malignant tumor (e.g. a breast tumor, a prostate tumor, or a carcinoma) which expresses a receptor which is recognized by the recombinant human alpha-fetoprotein (or fragment or analog thereof). Typically, such recombinant alpha-fetoprotein is produced in a prokaryotic cell (e.g., E. coli) and is unglycosylated.

Detectably-labelled recombinant human alpha-fetoprotein (or is a fragment or analof thereof) is useful for methods of imaging a neoplastic cell-containing region in a human patient in vivo. In general, the method involves: (a) providing a detectably-labelled molecule of recombinant human alpha-fetoprotein (or a fragment or analog thereof); (b) administering the molecule to the patient; (c) allowing the labelled molecule to bind and allowing unbound molecule to be cleared from the region; and (d) obtaining an image of the neoplastic cell-containing region. Preferably, the region is the breast or is the prostate. In other preferred embodiments, the region, without limitation, is liver tissue, is lung tissue, is spleen tissue, is pancreatic tissue, is brain tissue, is lymph tissue, or is bone marrow. Preferably, the image is obtained using dynamic gamma scintigraphy.

Detectably-labelled rec mbinant human alpha-fetoprotein (or a fragment or analog thereof) can also be used in a method for diagnosing a neoplasm in a mammal (e.g., a human patient). Such a method includes: (a) contacting the biological sample with the detectably-labelled molecule of recombinant human alpha-fetoprotein; and (b) detecting the label bound to the sample, where the detection of label above background levels is indicative that the patient has a neoplasm. Preferably, the method involves a biological sample including cells fixed and sectioned prior to the contacting step, and the label bound to the sample is bound to areas corresponding to the cell membrane of the cells. In preferred embodiments, the biological sample is from the breast or prostate of a human patient.

Detectably-labelled recombinant human alpha-fetoprotein (or fragment or analog thereof) can also be used in a method for detecting a neoplasm a mammal in vivo. Such a method includes: (a) administering a diagnostically effective amount of the detectably-labelled molecule of recombinant human alpha-fetoprotein; and (b) detecting the presence of the detectable label bound to a tissue of the mammal, where an amount of label above background levels is indicative of the presence of the neoplasm in the mammal.
In preferred embodiments, the method involves a human patient suspected of having a breast cancer, and the tissue is breast tissue. In other preferred embodiments, the method involves a human patient suspected of having a prostate cancer, and the tissue is prostate tissue. Preferably, the detectably labelled recombinant human alpha-fetoprotein is linked to a radionuclide (e.g., technetium-90) and the detection step is accomplished by radioimaging (e.g., dynamic gamma scintigraphy).

In another aspect, the invention features kits for detecting a neoplasm or any cell expressing a receptor which is recognized by recombinant human alpha-fetoprotein (or a fragment or analog thereof) *in vivo*, *in situ* or *in vitro*. In general, the kits include a recombinant human alpha-fetoprotein which is recognized by a neoplasm, and which may be detectably labeled. If the recombinant human alpha-fetoprotein is unlabelled, a second reagent containing a detectable label (e.g. a radionuclide such as technetium-90, iodine-125, iodine-131, or indium) is preferably included. Where the detectable label is an enzyme, the kit further includes a substrate reagent for the enzyme. The kit may also include a reagent for linking the detectable label to the recombinant alpha-fetoprotein. In another embodiment, the kit for detecting a neoplasm or any unwanted cell expressing a receptor which is recognized by recombinant human alpha-fetoprotein (or a fragment or analog thereof) includes a reagent containing an antibody which specifically binds the recombinant human alpha-fetoprotein and a reagent including a detectably labeled recombinant human alpha-fetoprotein that is specifically bound by the anti-alpha-fetoprotein antibody. Preferably, the recombinant human alpha-fetoprotein of the kit is produced in a prokaryotic cell (*E. coli*) and is unglycosylated.

The use of recombinant human alpha-fetoprotein for the treatment and diagnosis of cancer offers a number of advantages. For example, rHuAFP can be administered directly to a tumor site. Recombinant HuAFP can also be chemically defined and synthesized, and prepared in large quantities using the techniques of recombinant DNA, e.g., those which are described herein. Moreover, unlike conventional cancer chemotherapies and radiotherapies, recombinant human alpha-fetoprotein causes minimal side

effects such as nausea, vomiting, and neurotoxicity. Accordingly, relatively high doses of rHuAFP can be safely administered.

The diagnostic methods of the invention are advantageous since they allow for rapid and convenient diagnosis of a neoplasm. For example, the use of rHuAFP as a diagnostic agent (e.g., by radioimaging using scintigraphy) is especially advantageous for real time imaging of cancer for pre-surgical or intraoperative localization and for staging of a cancer, e.g., breast cancer, as well as during post-surgical examinations. The use of such diagnostic procedures permits non-invasive determination of the presence, location, or absence of a neoplasm which is advantageous for monitoring the condition of a patient.

In still other aspects, the invention features a cell culture medium including recombinant human alpha-fetoprotein or a cell-stimulating fragment or analog thereof. This aspect of the invention is based on my discovery that unglycosylated recombinant human alpha-fetoprotein made in a prokaryote (e.g., E. coli) is a cell proliferative agent, e.g., promotes the growth of bone marrow in vitro. Preferably, such recombinant human alpha-fetoprotein is produced in a prokaryotic cell (E. coli) and is unglycosylated.

Accordingly, this aspect of invention features a method of cell culture, the method including (a) providing a cell culture medium including recombinant human alpha-fetoprotein; (b) providing a cell; (c) and growing the cell in the medium, where the cell proliferates, and is maintained. Preferably, the cell is a mammalian cell. Examples of such mammalian cells include bone marrow cells (e.g., T cells, natural killer cell, lymphocyte), hybridomas, or a genetically-engineered cell line. Examples of other cells include

hemat poietic c lls such as stem cells, blast cells, progentior cells (.g., erythroid progenitor cells such as burst-forming units and colony-forming units), myeloblasts, macrophages, monocytes, macrophages, lymphocytes, T-lymphocytes, B-lymphocytes, eosinophils, basophils, tissue mast cells, megarkaryocytes (see e.g., *Best and Taylor's Physiological Basis of Medical Practice*, John B. West, ed., Williams & Wilkins, Baltimore). In other preferred embodiments the method involves ex vivo cell culture.

In another aspect, the invention features a method for inhibiting myelotoxcity in a mammal (e.g., a human patient) involving administering to the mammal a therapeutically effective amount of recombinant human alpha-fetoprotein or a myelotoxic-inhibiting analog or fragment thereof. Preferably, the recombinant human alpha-fetoprotein is produced in a prokaryotic cell (E. coli) and is unglycosylated.

In another aspect, the invention features a method of inhibiting suppression of bone marrow cell proliferation in a mammal, the method involving administering to the mammal an effective amount of recombinant alpha-fetoprotein or an anti-suppressive fragment or analog thereof. Preferably, the recombinant human alpha-fetoprotein is produced in a prokaryotic cell (e.g., E. coli) and is unglycosylated.

In another aspect, the invention features a method of promoting bone marrow cell proliferation in a mammal, involving administering to the mammal an effective amount of recombinant human alpha-fetoprotein or a cell-stimulating fragment or analog thereof. Preferably, the recombinant human alpha-fetoprotein is produced in a prokaryotic cell (e.g., E. coli) and is unglycosylated.

In another aspect, the invention features a method of preventing bone marrow cell transplantation rejection

in a mammal, involving administering to the mammal an effective amount of rec mbinant human alpha-fetoprotein or an anti-rejection fragment or analog thereof. Preferably, the recombinant human alpha-fetoprotein is produced in a prokaryotic cell (e.g., E. coli) and is unglycosylated. According to the methods of the invention, administration of rHuAFP (or a fragment or analog thereof) can be an effective means for promoting and boosting cell growth in vitro, ex vivo, or in vivo. Additionally, administration of the compounds of the invention can also be an effective means of preventing or treating or ameliorating myleotoxcemia in a mammal.

The use of rHuAFP (or a fragment or analog thereof) as a principal component of tissue culture media is advantageous since there is little potential for contamination with pathogens.

By "human alpha-fetoprotein" is meant a polypeptide having substantially the same amino acid sequence as the protein encoded by the human alpha-fetoprotein gene as described by Morinaga et al., Proc. Natl. Acad. Sci., USA 80: 4604 (1983). The method of producing recombinant human alpha-fetoprotein in a prokaryotic cell is described in U.S. Pat. No. 5,384,250, and according to the methods described herein.

By "expression control element" is meant a nucleotide sequence which includes recognition sequences for factors that control expression of a protein coding sequence to which it is operably linked. Accordingly, an expression control element generally includes sequences for controlling both transcription and translation, for example, promoters, ribosome binding sites, repressor binding sites, and activator binding sites.

By "substantially the same amino acid sequence" is meant a polypeptide that exhibits at least 80% homology

with naturally occurring amino acid sequence of human alpha-fet pr tein, typically at least about 85% homology with the natural human alpha-fetoprotein sequence, more typically at least about 90% homology, usually at least about 95% homology, and more usually at least about 97% homology with the natural human alpha-fetoprotein sequence. The length of comparison sequences will generally be at least 16 amino acids, usually at least 20 amino acids, more usually at least 25 amino acids, typically at least 30 amino acids, and preferably more than 35 amino acids.

Homology, for polypeptides, is typically measured using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Protein analysis software matches similar sequences by assigning degrees of homology to various substitutions, deletions, substitutions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

As used herein, the term "substantially pure" describes a protein or polypeptide which has been separated from components which naturally accompany it. Typically, a protein of interest is substantially pure when at least 60% to 75% of the total protein in a sample is the protein of interest. Minor variants or chemical modifications typically share the same polypeptide sequence. A substantially pure protein will typically comprise over about 85 to 90% of the protein in sample, more usually will comprise at least about 95%, and preferably will be over about 99% pure. Normally, purity

is measured on a chromatography c lumn, polyacrylamide gel, or by HPLC analysis.

A protein is substantially free of naturally associated components when it is separated from the native contaminants which accompany it in its natural state. Thus, a protein which is chemically synthesized or produced in a cellular system different from the cell from which it naturally originates will be substantially free from its naturally associated components. Thus the term can be used to describe polypeptides and nucleic acids derived from eukaryotic organisms which have been synthesized in E. coli and other prokaryotes.

The present invention provides for substantially pure human alpha-fetoprotein. Various methods for the isolation of human AFP from biological material may be devised, based in part upon the structural and functional properties of human alpha-fetoprotein. Alternatively, anti-AFP antibodies may immobilized on a solid substrate to generate a highly specific affinity column for purification of human AFP.

Besides substantially full-length polypeptides, the present invention provides for biologically active recombinant fragments or analogs of human alpha-fetoprotein. For example, fragments active in ligand binding or immunosuppression.

The natural or synthetic DNA fragments coding for human alpha-fetoprotein or a desired fragment thereof will be incorporated into DNA constructs capable of introduction into and expression in cell culture. DNA constructs prepared for introduction into such hosts will typically include an origin of replication which can be utilized by the host cell, a DNA fragment encoding the desired portion of human alpha-fetoprotein, transcription and translational initiation regulatory sequences operably linked to the alpha-fetoprotein encoding

segment, and transcripti nal and translational termination regulatory sequences operably linked to the alpha-fetoprotein encoding segment. The transcriptional regulatory sequences will typically include a heterologous promoter which is recognized by the host. The selection of an appropriate promoter will depend upon the host, but promoters such as the trp, tac and phage promoters, tRNA promoters and glycolytic enzyme promoters may be used under appropriate circumstances (Sambrook et al. eds., *Molecular Cloning: Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY 1989). In some instances it may be desirable to include appropriately positioned recognition sequences for factors capable of regulating transcription in the host cell (e.g., the lac repressor of E. coli). Commercially available expression vectors, which include the replication system and transcriptional and translational regulatory sequences together with convenient sites for the insertion of a DNA fragment encoding the gene to be expressed may be used.

The various promoters, transcriptional, and translational described above are generally referred to as an "expression control element."

It is also possible to integrate a DNA fragment encoding all or part of human AFP into the host cell's chromosome.

The vectors containing the DNA segments of interest can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host (Sambrook et al., supra). The term "transformed cell" is meant to also include the progeny of a transformed cell.

Prokaryotic hosts useful for high level expression of recombinant proteins include: various strains of E. coli, Bacillus subtilis, and Pseudomonas.

The method of the invention provides a means by which to generate large quantities of human alpha-fetoprotein having biological activity. AFP produced according to the method of the invention has biological activity despite the fact that it is not modified in the same fashion as naturally occurring human AFP.

By "immune cell anti-proliferative" is meant capable of inhibiting the growth of an undesirable immune cell (e.g., an autoreactive T cell as measured using the assays described herein).

By "neoplasm" is meant any unwanted growth of cells serving no physiological function. In general, a cell of a neoplasm has been released from its normal cell division control, i.e., a cell whose growth is not regulated by the ordinary biochemical and physical influences in the cellular environment. In most cases, a neoplastic cell proliferates to form a clone of cells which are either benign or malignant. Examples of neoplasms include, without limitation, transformed and immortalized cells, tumors, and carcinomas such as breast cell carcinomas and prostate carcinomas.

By "therapeutically effective amount" is meant a dose of unglycosylated recombinant human alpha-fetoprotein or an anti-neoplasm fragment or analog thereof capable of inhibiting the proliferation of a neoplasm or is capable of inhibiting autoreactive immune cell proliferation or stimulating the proliferation of a cell (e.g., a bone marrow cell).

By "diagnostically effective amount" is meant a dose of detectably-labelled recombinant human alpha-fetoprotein or a detectably-labelled fragment or analog thereof that can be detected within a targeted region in a mammal (e.g., a human patient).

By "cell-stimulating" is meant increasing cell proliferation, increasing cell division, promoting cell

differentiation and/or development, r promoting cell longevity.

By "my lotoxic-inhibiting" is meant inhibiting myeloablation.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Detailed Description

The drawings will first be described.

Drawings

Fig. 1 is the nucleotide sequence (SEQ ID NO: 4) and deduced amino acid sequence (SEQ ID NO: 5) of the cDNA encoding human alpha-fetoprotein.

Fig. 2 is the 10% SDS-PAGE analysis of rHuAFP Fragment I (SEQ ID NO: 11) (Lane A, MW marker; Lane B, natural human alpha-fetoprotein (AFP); Lane C, unpurified rHuAFP; Lane D, rHuAFP Fragment I, and Lane E, rHuAFP (amino acids 1- 590 of Fig. 1, SEQ ID NO: 5).

Fig. 3 is a bar graph showing the inhibition of human AMLR by E. coli-derived rHuAFP and domain fragments.

Fig. 4 is a series of graphs (**Figs. 4A-4D**) showing the purity and biochemical characteristics of baculovirus- and E. coli-derived rHuAFP using polyacrylamide gel electrophoresis and column chromatography. **Fig. 4A** is a 10% non-denaturing alkaline polyacrylamide gel showing the purity of rHuAFP. Mouse amniotic fluid proteins (transferrin, AFP and albumin) are shown in lane 1, natural HuAFP (lane 2), baculovirus-derived rHuAFP (lane 3), and E. coli-derived rHuAFP (lane 4). **Fig. 4B** is a 10% sodium dodecyl sulfate-polyacrylamide gel showing the purity of rHuAFP produced using baculovirus and E. coli expression systems. Molecular weight markers are shown in lane 1, natural

HuAFP, baculovirus- and E. coli-derived rHuAFP are shown in lanes 2, 3, and 4, respectively. Fig. 4C is a series of FPLC chromatograms of natural HuAFP, baculovirus-derived and E. coli-derived rHuAFP eluted on a MonoQ anion exchange column. The superimposed chromatograms identify natural HuAFP (Chromatogram 1), baculovirus- and E. coli-derived rHuAFP (Chromatograms 2 and 3, respectively). Fig. 4D is a series of HPLC chromatograms obtained by passing 50 μg of natural and rHuAFP by passing through a reverse phase Delta Pak C18 column (Waters) and eluting with a gradient of 0-100% acetonitrile in 0.1% TFA. The superimposed chromatograms identify natural HuAFP (Chromatogram 1) and baculovirus- and E. coli-derived rHuAFP (Chromatograms 2 and 3, respectively).

Fig. 5 is a bar graph showing that monoclonal anti-natural HuAFP antibodies block immunosuppression of the AMLR by rHuAFP produced using baculovirus and E. coli expression systems. Immunosuppression by rHuAFP produced using baculovirus and E. coli expression systems was significant ($p<0.002$) and blocking of rHuAFP-mediated immunosuppression by the AMLR by monoclonal anti-natural HuAFP (à AFP) antibodies was also significant ($p<0.03$). AMLR cultures were set up with 2×10^5 responding T cells with 2.5×10^5 irradiated autologous non-T cells in the presence or absence of protein, harvested at 144 hours, and autoproliferation was measured by the amount of ^3H-thymidine incorporated by autoreactive T cells. Blocking of the autoproliferative effects of rHuAFP was carried out by adding murine anti-human AFP monoclonal antibodies at a dilution of 1/8 (125 μg/ml) to AMLR cultures suppressed by 100 μg/ml baculovirus derived (diagonal bars) and by 100 μg/ml of E. coli-derived (open bars) rHUAFP. Control cultures consisted of the AMLR in the

presence f 1/8 dilution of anti-human AFP (à AFP) mon clonal antibodies.

Fig. 6 is a series of bar graphs (Figs. 6A and 6B) showing the effects of rHuAFP-mediated immunosuppression using human AMLR (Fig. 6A) and PBL (Fig. 6B) assays. Fig. 6A shows the results of autologous mixed lymphocyte reaction (AMLR) prepared by co-culturing 250,000 T cells with an equivalent amount of autologous irradiated non-T lymphocytes. Recombinant HuAFP preparations derived from E. coli and baculovirus expression systems and albumin were added at a concentration of 100 μg/ml at the initiation of culture. Proliferative responses were measured at 144 hours by ^{3}H-thymidine incorporation. Fig. 6B shows the results of PBLs (2 x 10^{5}) stimulated with 1 μg/ml ConA which were cultured in RPMI medium supplemented with only 2 mg/ml albumin for 48 hrs. Albumin and rHuAFP derived from E. coli and baculovirus were added to the initiation of the cultures at a concentration of 100 μg/ml. Proliferative responses were measured as the amount of ^{3}H-thymidine incorporated during DNA synthesis. The SEM were determined to represent less than 5% of the value of the mean.

Fig. 7 is a plasmid map of pVT-PlacZ.

Fig. 8 is a series of graphs showing the inhibitory effects of the rHuAFP on kinetics of T cell activation (Fig. 8A) and the dose-response relationship of rHuAFP on autoproliferating T cells (Fig. 8B). Fig. 8A is a graph showing proliferative responses over a 4 day time course of cells cultured in the absence (▽) and in the presence of 100 μg/ml (▼) rHuAFP. (●) denotes the background proliferation of the responder cell population cultured separately. Recombinant HuAFP-mediated suppression on the AMLR over the time course was significant ($p<0.01$). Fig. 8B is a graph showing the inhibition of autoproliferating T cells at 144 hours with

amounts of rHuAFP ranging from 6-100 μg/ml (▼). (▽) denotes the control response of the reaction in the absence of protein. Inhibition of autoreactive T cells by rHuAFP in the range of 12.5-100 μg/ml is significant ($p<0.005$).

Fig. 9 is a bar graph showing the effect of rHuAFP on estrogen-stimulated post-confluent growth of MCF-7 breast cancer cells.

Fig. 10 is a bar graph showing murine bone marrow proliferation in serum-free RPMI medium in the presence or absence of both 400 μg/ml rHuAFP and 5μg/ml transferrin.

Expression of Recombinant Human Alpha-Fetoprotein

Construction of a cDNA Library

A cDNA library was constructed with size-fractionated cDNA (0.5-3 kb) prepared from poly(A)$^+$ RNA isolated from liver cells (~3 grams wet weight) of a 4.5 months old human abortus. (Alternatively, a fetal cDNA library may be obtained from Clontech Laboratories, Inc., Palo Alto, CA.) Total RNA was prepared by the guanidium thiocyanate method (Chirgwin et al., Biochemistry 18:5294, 1979), and mRNA was selected by oligo(dT)-cellulose chromatography (Collaborative Research, Bedford, MA) (*Current Protocols in Molecular Biology*, Ausubel et al., eds., Wiley Interscience, New York, 1989). cDNA was synthesized using the Librarian II cDNA synthesis kit (Invitrogen, San Diego, CA) and fractionated on a 1% agarose gel. Fragments of 0.5 to 3 kb were extracted and ligated to vector pTZ18-RB (Invitrogen), and used to transform competent E. coli DH1αF' (Invitrogen). Colony lifts were performed with Colony/Plaque Screen filters (DuPont, Wilmington, DE), and the transferred bacterial colonies were lysed and denatured by incubation in a solution of 0.5M NaOH, 1.5M NaCl for 10 min. The filters were washed for 5 min in

1.5M NaCl, 0.50M Tris-HCl (pH 7.6), and air dried. Filters were then washed 5 times in chloroform, soaked in 0.3M NaCl to remove cellular debris, and then air dried. The DNA was fixed to the nitrocellulose by baking at 80° under vacuum for 2 hrs. The baked filters were prehybridized for 3 hr at 37°C in 6X SSC (1X SSC = 150mM NaCl, 15mM sodium citrate [pH 7.0]), 1X Denhardt's solution (0.2 g/l polyvinylpyrrolidone, 0.2 g/l BSA, 0.2 g/l Ficoll 400), 0.05% sodium pyrophosphate, 0.5 % SDS, and 100 µg/ml E. coli DNA. Hybridization was performed for 18-24 hr at 37°C in the same solution without SDS, containing 1-2 x 10^6 cpm/ml of two oligonucleotides ^{32}p-labelled by 5'-end phosphorylation (Current Protocols in Molecular Biology, supra). The sequence of the oligonucleotides used for probing the library: 5'-TGTCTGCAGGATGGGGAAAAA-3' (SEQ ID NO: 1) and 5'-CATGAAATGACTCCAGTA-3' (SEQ ID NO: 2), correspond to positions 772 to 792 and positions 1405 to 1422 of the human AFP coding sequence respectively. Filters were washed twice for 30 min at 37°C with 6X SSC, 0.05% sodium pyrophosphate and once for 30 min at 48°C in the same solution. Dried filters were exposed to Kodak XAR films in the presence of Du Pont Cronex Lightning Plus intensifier screens for 24-48 hr to identify positive clones. Positive clones were isolated, amplified, and subjected to Southern blot analysis (Current Protocols in Molecular Biology, supra). Briefly, purified DNA was hydrolyzed with the appropriate restriction enzymes, and the resulting fragments were resolved on a 1% agarose gel. The DNA was then transferred to a nitrocellulose membrane. Hybridization conditions were as described above except that a third ^{32}p-labelled oligonucleotide (5'-CATAGAAATGAATATGGA-3' (SEQ ID NO: 3), representing positions 7 to 24 of the human AFP coding region) was used in addition to the other two probes described above.

Fiv positive clones wer identified among the 3,000 colonies screened. One clone, pLHuAFP, was used in the construction described below.

Construction of Full Length Human AFP cDNA

A construct containing a translation initiation codon followed by the human AFP coding sequence and a translation termination codon was created using the following five DNA fragments.

Fragment 1: Two unphosphorylated oligonucleotides were annealed to form a double-stranded DNA molecule consisting of a 5'-end cohesive EcoRI recognition site, followed by an ATG initiation codon and the first 60 bp of the human AFP cDNA up to and including the PstI restriction site located at position 60 in the coding sequence (In this scheme, nucleotide 1 is the first nucleotide of the first codon (Thr) in the mature protein and corresponds to nucleotide 102 of Morinaga et al., supra). This fragment was ligated to pUC119 (pUC19 with the intergenic region of M13 from HgiA I at 5465 to AhaII at 5941 inserted at the Nde I site of pUC19) linearized with Eco RI and Pst I. The resulting DNA was amplified in E. coli NM522 (Pharmacia, Piscataway, NJ) The EcoRI-PstI insert was recovered by enzymatic digestion of the recombinant plasmid followed by electrophoretic separation on a 5% polyacrylamide gel and isolation from the gel.

Fragment 2: A 97 bp human AFP cDNA fragment (positions 57 to 153) was obtained by digesting pLHuAFP with PstI and NsiI and gel purifying as described above. This clone contains the entire coding region of human AFP as well as 5' and 3' untranslated sequences.

Fragment 3: A 224 bp human AFP cDNA fragment (positions 150 to 373) was obtained by digesting pLHuAFP with NsiI and AlwNI and purifying as described above.

Fragment 4: A 1322 bp human AFP cDNA fragment (positions 371 to 1692) was btained by dig sting pLHuAFP with AlwNI and StyI and purifying as described above.

Fragment 5: Two unphosphorylated oligonucleotides were annealed to form a 86 bp double-stranded DNA contains the human AFP sequence from position 1693 in the StyI site to the TAA termination codon that ends the AFP coding region at position 1773, followed by a cohesive BamHI site. This synthetic DNA was used without any further manipulations.

pBlueScript (StrataGene, La Jolla, CA) was completely hydrolyzed with EcoRI and BamHI, and added to ligation mixture containing the five purified fragments described above. A control ligation contained only the linearized pBluescript. Portions of these two ligation mixtures were used to transform competent E. coli DH5α (GIBCO/BRL, Grand Island, NY). Recombinant plasmids were isolated from several transformants and screened by extensive restriction enzyme analysis and DNA sequencing. One recombinant plasmid was selected and termed pHuAFP. It was used for subsequent insertion of the human AFP gene into several expression vectors. pHuAFP includes a unique EcoRI-BamHI fragment that contains the complete coding sequence for human AFP in addition to an ATG start codon at the 5'-end and a TAA stop codon at the 3'-end.

AFP Expression Vectors

Successful high-level synthesis of human AFP in E. coli was achieved in three different expression systems. The TRP system gave direct expression. The RX1 system yielded a fusion protein containing 20 amino acids encoded by trpE and vector sequences. The MAL system expressed AFP fused to the malE gene product, a 42 kd maltose-binding protein.

TRP Expression System: The 1186 bp EcoRI-BamHI AFP encoding fragment of pHuAFP was cloned into the

expression vector pTrp4 (Olsen et al., J. Biotechnol. 9:179, 1989) downstream of the trp promoter and a modified ribosome-binding site.

Briefly, pHuAFP was digested with EcoRI and BamHI, and the ends were filled using Klenow polymerase. The 1186 bp AFP fragment was then gel purified. pTrp4 was ClaI digested, the ends were filled using Klenow polymerase, and the linearized vector was gel purified. The 1186 bp AFP fragment and pTrp4 backbone were ligated and used to transform competent E. coli of the following strains: DH5α, BL21 (F.W. Studier, Brookhaven National Laboratory, Upton, NY), SG927 (American Type Culture Collection, Rockville, MD: Acc. No. 39627), SG928 (ATCC Acc. No. 39628), and SG935 (ATCC Acc. No. 39623).

RX1 Expression System: Human AFP cDNA was cloned into the expression vector pRX1 (Rimm et al., Gene 75:323, 1989) adjacent to the trp promoter and in the translation frame of TrpE. The human AFP cDNA was excised from pHuAFP by digestion with EcoRI and BamHI and cloned into suitably treated pRX1 (BioRad Laboratories, Hercules, CA). The E. coli strains described above and CAG456 (D.W. Cleveland, Johns Hopkins University, Baltimore, MD) were then transformed with the final plasmid construction identified as pRX1/HuAFP.

MAL Expression System: AFP cDNA was into inserted in the expression vector pMAL (New England Biolabs, Inc., Beverly, MA) under control of the tac promoter and in the translation frame of MalE. Briefly, pHuAFP was hydrolysed with BamHI and the ends made blunt using Klenow polymerase. The human AFP cDNA was released from the rest of the plasmid DNA by EcoRI digestion and then gel purified. The purified fragment was ligated to appropriately digested pMAL-C. A correctly oriented recombinant plasmid, designated pMAL/HuAFP, was used to

transform E. coli DH5α, TBI (New England Biolabs) and SG935.

The AFP coding region used in the construction of the three expression vectors was sequenced and found to encode full length AFP.

Expression of AFP in E. coli

Bacterial cultures were incubated at 30°C or 37°C with aeration. Overnight cultures of E. coli were grown in LB medium supplemented with the appropriate antibiotics as required (Tetracycline-HCl was at 50 μg/ml, and ampicillin-Na was at 100 μg/ml).

TRP and RX1 Expression Systems: The trp promoter was induced under tryptophan starvation conditions. Induction was performed in M9CA medium prepared as follows: 1 g Casamino acids (Difco Laboratories, Detroit, MI), 6 g Na_2HPO_4, 3g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl is added to one liter milli-Q water (Millipore Corp., Bedford, MA), the pH adjusted to 7.4 and the solution autoclaved. The cooled medium is made 2mM $MgSO_4$, 0.1mM $CaCl_2$, and 0.2% glucose. After a 100-fold dilution of an overnight culture in M9CA supplemented with antibiotics, the cells were grown at 30°C to A_{550} of 0.4, harvested by centrifugation, and stored as pellets at -20°C.

MAL Expression System: The tac promoter was induced with the gratuitous inducer IPTG. Overnight cultures were diluted 100-fold in LB medium supplemented with antibiotics, and the cells grown at 37°C to A_{550} of 0.4. IPTG was then added to a final concentration of 0.3mM, and the bacteria incubated an additional 2 hr. The cells were then harvested by centrifugation and stored as pellets at -20°C.

Detection of AFP Expressed in E. Coli

Analytical studies were performed to determine the expression and behavior of recombinant AFP. Cell pellets were either suspended in SDS-lysis solution (0.16M Tris-

HCl [pH 6.8], 4% w/v SDS, 0.2M DTT, 20% glycerol, 0.02% bromophenol blue), b il d for 5 min, and us d for analysis by SDS-PAGE or suspended in a lysis buffer consisting of 10 mM Na_2HPO_4, 30mM NaCl, 0.25% Tween 20, 10mM EDTA, 10mM EGTA and incubated with 1 mg/ml lysozyme at 4°C for 30 min prior to sonication in pulse mode for 3 x 1 min at 50% power (Sonics and Materials, Danbury, CT: model VC300 sonifier). The lysate was centrifuged at 10,000g for 20 min, and the supernatant containing soluble protein was decanted in a separate test tube and frozen at -20°C until used. The pellet containing insoluble protein was resuspended in SDS-lysis buffer, boiled for 5 min and kept at -20°C until used. Total protein released in SDS-lysis buffer, as well as soluble and pellet fractions were analyzed by SDS-PAGE and immunological detection following western blot transfer. In these studies Coomassie blue stained gels were routinely scanned with a video densitometer (BioRad, model 620). This allowed a qualitative assessment of the amount of recombinant AFP produced as a percentage of total cellular protein.

Purification of AFP Expressed in the TRP System

All procedures were carried out at 4°C, unless otherwise stated. Each frozen cell pellet from a one liter culture was resuspended in 25 ml of lysis buffer A, 50mM Tris-HCl [pH 7.5], 20% sucrose, 100 µg/ml lysozyme, 10 µg/ml PMSF), and incubated for 10 min. EDTA was added to a final concentration of 35mM, and the extract allowed to stand a further 10 min. Following the addition of 25 ml of lysis buffer B (50mM Tris-HCl [pH 7.5], 25mM EDTA, 0.2% Triton X-100), the lysate was incubated an additional 30 min. The cell lysate was centrifuged at 12,000g for 20 min, and the precipitate containing the recombinant AFP was washed twice with 50 ml of wash buffer (50mM Tris-HCl [pH 8.0], 10mM EDTA, 0.2% Triton X-

100), followed each time by centrifugation as above. The precipitate was dissolved in 50 ml of denaturation buffer (0.1M K_2HPO_4 [pH 8.5], 6M guanidine-HCl, 0.1M 2-mercaptoethanol), sonicated, and then mixed on a Nutator (Clay Adams) for 4 hr. The solubilized extract was diluted 50-fold in 50mM Tris-HCl, 100mM NaCl, 1mM EDTA, and the recombinant AFP protein allowed to renature for 24 hr. This 50-fold dilution step is important because prior to dilution AFP appears to be microaggregated. Subsequent to dilution and reconcentration, AFP is not aggregated. The solution was concentrated 100-fold on YM10 membranes using an Amicon filtration unit, and clarified through a Millex 0.22 μm membrane filter (Millipore). The recombinant AFP was further purified at room temperature on a Mono Q column (Pharmacia) equilibrated in 20mM Tris-HCl (pH 8.0) with bound proteins eluted using a linear gradient of 0-100% 1M NaCl, 20mM Tris-HCl (pH 8.0). Fractions were analyzed by SDS-PAGE, APAGE, and Western blotting.

These general techniques of polypeptide expression and purification can also be used to produce and isolate useful human alpha-fetoprotein fragments or analogs (described below).

Polyacrylamide Gel Electrophoresis and Western Immunodetection Procedures

SDS-PAGE in discontinuous buffer system and alkaline-PAGE were performed according to Hames et al. (*Gel Electrophoresis of Proteins: A Practical Approach*, IRL Press, London, 1981) using the mini-Protean electrophoresis apparatus (BioRad). Immunological detection of recombinant human AFP following SDS-PAGE or APAGE was accomplished by soaking the gels in transfer buffer (12.5mM Tris-HCl, 96mM glycine, 20% methanol [pH 8.2]) for 15 min. Individual gels were then layered with an Immobilon PVDF membrane (Millipore) and sandwiched

b tween th two electrode grids of the mini-Protean transfer device (BioRad), with the gels adjacent to the cathode. The system was immersed in transfer buffer, and a 150 mA current was applied for 2 hr. Unreacted sites on the Immobilon PVDF sheets were blocked in 20mM Tris-HCl (pH 7.5), 500mM NaCl, 3% gelatin for 1 hr. Rabbit anti-human AFP antiserum and goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase (BioRad) were used as the primary and the secondary antibodies, respectively. The alkaline phosphatase activity was detected using 5-brome-4-chloro-3-indolyl phosphate and p-nitroblue tetrazolium (Bio-Rad).

Quantitation of AFP Expression

Recombinant human AFP was quantitated using a human AFP ELISA kit (Abbott Laboratories, Chicago, IL).

AFP yield was estimated by scanning silver stained gels. When SG935 cells are transformed with the AFP encoding plasmid that employs the Trp expression system, AFP represents 2 to 5% of total cellular E. coli protein (approximately 3-7 mg AFP per liter of culture). As described above, most AFP in the initial extract is insoluble. The above-described resolubilization procedure permits 50-60% recovery of AFP in the form of stable, semi-purified, monomeric AFP (approximate yield 50 mg/20 l of E. coli). This can be further purified to yield 25 mg of pure monomeric AFP.

N-Terminal Analysis

Automatic Edman degradations were performed using a Porton protein/peptide gas phase microsequencer with an integrated customized microbore HPLC to optimize sequence. Protein sequence analysis was aided by the use of selected programs within the PC/Gene software package (Intelligenetics).

Cloning, Expr ssion, and Purification of HuAFP Using A Baculovirus Expressi n System

Recombinant baculovirus expressing HuAFP (or a fragment or analog therof) is constructed according to standard methods known in the art (see, e.g., U.S. Pat. No. 4,745,051). This process generally involves two steps. The gene to be expressed, e.g., rHuAFP or a fragment or analog thereof (described infra), is first cloned into a plasmid transfer vector downstream from a baculovirus promoter that is flanked by baculovirus DNA derived from a nonessential locus, e.g., the polyhedrin gene. This plasmid is then introduced into insect cells along with circular wild-type genomic DNA for homologous recombination to occur. Resulting recombinant progeny are then screened, e.g., using sequential plaque assays to purify recombinant virus away from the nonrecombinant parental strain. Viral amplification is also generally necessary to obtain sufficient virus for protein expression. Recombinant virus are plaque purified and their DNA structure confirmed using standard methods well known in the art.

The rHuAFP cDNA fragment was isolated from plasmid pI18 with EcoRI/BamHI and purified using Geneclean as described infra. The cloning and expression of the HuAFP cDNA in baculovirus was performed using plasmid pVT-PLacZ. Human AFP cDNA was cloned into plasmid pVT-PlacZ (Fig. 7) in frame with the 3'-end of the insect derived melittin signal peptide sequence. The baculovirus vector pVT-PlacZ was modified by replacing the multiple cloning site with the oligonucleotide 5'-GATCTAGAATTCGGATCCGGT-3' (SEQ ID NO: 21) and its complementary fragment, containing EcoRI and BamHI restriction sites in the 5' and 3' direction, reducing the number of non-AFP coding nucleotides between the melittin signal peptide cleavage site and the location of the AFP cDNA insert at the EcoRI

ndonuclease sequence. The insert was th n ligated into the modified pVT-PLacZ vect rs at the Ec RI and BamHI DNA sequences.

The generation of recombinant baculovirus containing rHuAFP coding sequences was performed according to standard techniques. Accordingly, purified recombinant baculovirus containing the coding sequences of HuAFP were generated by co-transfection of the pVT-PLacZ transfer vectors and wild-type baculovirus, followed by two rounds of plaque purification. Sf9 insect cells seeded at a density of 1 x 10^6 cells/ml in 500 ml spinner flasks were infected with recombinant baculovirus in serum-free Grace medium at a multiplicity of infection of 5. The supernatant containing secreted rHuAFP was harvested and cells were removed by centrifuging at 200 x g. The rHuAFP containing medium was concentrated 10-20 fold by ultrafiltration with a YM30 Amicon membrane, dialysed overnight against PBS and then applied to a Con A lectin column (Pharmacia). Bound rHuAFP was eluted with 0.4M methyl α-D mannopyranoside and was purified by elution from Mono Q resin during a linear gradient from 0-100% 1M NaCl in 20 mM phosphate buffer, pH 8.0. Recombinant HuAFP was characterized according to methods well known in the art.

I found that baculovirus produced rHuAFP represented approximately 20% of the total proteins secreted into serum-free medium by the Sf9 insect cells. This AFP was also found to be monomeric as analyzed by non-reducing alkaline PAGE. The majority of the baculovirus-derived HuAFP bound to immobilized Con A. This resulted in effective removal of more than 90% of contaminating proteins which were nonadhered to lectin columns. Final purification of the baculovirus derived rAFP preparations was accomplished by eluting protein with 270-310 mM NaCl from MonoQ beads, yielding a single

polypeptide with an apparent molecular mass of approximately 68 kD. We obtained at least 1mg of purified protein was obtained per liter of growth culture.

The baculovirus-derived rHuAFP molecular weight is similar to the natural human molecule (Fig. 4B). This finding, in addition to the binding of baculovirus-derived rHuAFP and the observed non-adherence of *E. coli*-derived rHuAFP to the ConA column, indicates that the degree of glycosylation of the BrAFP is expected to be less than that of the native molecule, since Sf9 cells infected with recombinant baculovirus have been documented to be deficient in their ability to carry out complex glycosylation normally observed with higher eukaryotic derived proteins. Purity of the isolated baculovirus-derived rHuAFP was verified as a single band on APAGE and SDS-PAGE (Figs. 4A & 4B), and as a sole peak on FPLC and HPLC chromatograms as shown in Figures 4C and 4D, respectively. N-terminal sequencing further confirmed the identity of pure rHuAFP. The N-terminal sequence of the rHuAFP was as follows: Asp-Leu-Glu-Phe-Met-Thr-Leu-His-Arg-Asn (SEQ ID NO: 22). Western blot analysis of serum free supernatants from recombinant baculovirus infected Sf9 cells detected a single immunoreactive band with monospecific anti-HuAFP Ab that was absent form the supernatant of uninfected or wild-type virus-infected Sf9 cells.

Experiments were performed to evaluate the biological activity of the baculovirus produced HuAFP according to methods known in the art. For example, the immunosuppressive activity of 100 µg/ml of baculovirus-produced HuAFP was assessed by its ability to suppress human AMLR as described above. As shown in Figs. 5 and 6A, baculovirus-derived rHuAFP inhibited the

proliferative response of autoreactive lymphocytes stimulat d by autologous non-T cells at 144 hours. The addition of an identical amount of human serum albumin failed to diminish lymphoproliferative responses.

To demonstrate that rHuAFP was the substrate resposnsible for the inhibition of autoproliferating T cells, blocking of the rHuAFP-mediated suppression of the AMLR was performed using commercial murine anti-human AFP monoclonal antibodies (MAb). As shown in Fig. 5, suppression of proliferating autoreactive T cells by 100 μg/ml E. coli- and baculovirus-derived rHuAFP was completely blocked by anti-HuAFP MAb. The addition of 100 μg/ml of HSA did not diminish the AMLR response and the presence of MAb alone in the reaction culture was without any effect.

In addition, we tested the biological activity of rHuAFP to suppress the mitogen induced proliferation of peripheral blood lymphocytes (PBL) in RPMI tissue culture media supplemented only with 2 mg/ml purified human albumin (ICN, Mississauga, ON). As shown in Table II (below) and Fig. 6B, suppression of ConA stimulated PBLs occurred with 100 μg/ml rHuAFP whereas the same concentration of albumin was ineffective.

Other rHuAFPs (e.g., rHuAFP (Amino acids 1(Thr) - 590 (Val); SEQ ID NO: 5); Domain I (Amino acids 1 (Thr) - 197 (Ser), SEQ ID NO: 6; Domain II (Amino acids 198 (Ser) - 389 (Ser), SEQ ID NO: 7); Domain III (Amino acids 390 (Gln) - 590 (Val), SEQ ID NO: 8); Domain I + II (Amino acids 1 (Thr) - 389 (Ser), SEQ ID NO: 9); Domain II + III, (Amino acids 198 (Ser) - 590 (Val), SEQ ID NO: 10); rHuAFP Fragment I (Amino acids 266 (Met) - 590 (Val), SEQ ID NO: 11) can be produced using the above described baculovirus expression system according to standard methods, e.g., any of the methods described herein.

In one working example, the vector pVT-PLacZ/HuAFP (amino acids 1 - 590) is constructed by inserting the cDNA for HuAFP into pVT-P10, an intermediate vector in the construction of pVT-PLacZ (Richardson et al., (1992) Engineering Glycoproteins for secretion using the baculovirus expression system. *In*: *Baculovirus and Recombinant Protein Production Processes*, eds., J.M. Viak, E.-J. Schlaeger, and A.R. Bernard, Editiones Roche, Basel, Switzerland. pp. 67-73.). The pVT-P10 vector is digested with BamHI, followed by incubation with mung bean Nuclease (New England Biolabs, Mississauga, Ont.). The vector is then further hydrolyzed with EcoRl downstream of the blunt-end BamHl site to facilitate directional cloning of the HuAFP cDNA. The rHuAFP cDNA encoding amino acids 1-590 is obtained by PCR amplification, employing the following oligonucleotide primers: (5'-AAAAAACTCGAGATACACTGCATAGAAATGAA-3'; SEQ ID NO:23), containing an Xhol site and (5'-AAAAAAGAATTCTTAAACTCCCAAAGCAGCACG-3'; SEQ ID NO:24), containing an EcoRl site, and plasmid pl18 as the template DNA containing the coding region of HuAFP. The PCR reaction is performed according to standard methods, e.g., in a reaction mixture containing 34 μL H_2O, 10μL 10 x reaction buffer, 20 μL dNTP, 2μL DNA template, 10 μL 10 pmol/μL 5' primer, 10 μL 10 pmol/μL 3' primer, 1 μL glycerol, 10 μL DMSO, and 1 μL *Pfu* polymerase. Annealing, extension, and denaturation temperatures are also performed according to standard contions, e.g., 50°C, 72°C, and 94°C, respectively, for 30 cycles, using the GeneAmp PCR System 9600 (Perking Elmer Cetus). DNA from the PCR reaction is purified using the Genaclean kit (Bio 11 Inc., LaJolla, CA). The rHuAFP cDNA fragment is first digested with Xhol followed by treatment with mung bean nuclease. Next, the HuAFP cDNA is digested with EcoRl to facilitate directional cloning into pVT-p10.

The PCR-produced rHuAFP cDNA is ligated into the blunt ended 5' BamHl site and into the 3' EcoRl site. The β-galactosidase gene containing at the 3'end a polyadenylation site from SV40, isolated from the vector pJV-Nhel (Vialard et al., (1990) Synthesis of the membrane fusion and hemagglutinin proteins of Measles Virus, using a novel baculovirus vector containing the β-galactosidase gene, J. Virology 64: 37-50) by using the restriction enzyme BamHl, is then inserted into the compatible BgIII, producing the final construct: pVT-PlacZ/HuAFP (containing amino acids 1-590). Such a construct is then used for expressing rHuAFP (amino acids 1-590).

Fragments and Analogs

The invention includes biologically active fragments of rHuAFP. A biologically active fragment of rHuAFP is one that possesses at least one of the following activities: (a) directs a specific interaction with a target cell, e.g., binds to a cell expressing a receptor which is recognized by rHuAFP (e.g., the membrane of a cancer cell such as an MCF-7 or a bone marrow cell); (b) halts, reduces, or inhibits the growth of a neoplasm or an autoreactive immune cell (e.g., binds to a cell surface receptor and imparts an anti-proliferative signal); stimulates, increases, expands, or otherwise causes the proliferation of a cell such as a bone marrow cell (e.g., binds to a cell surface receptor a proliferative or stimulating-signal); or blocks or inhibits or prevents an immunopathologic antibody reaction. The ability of rHuAFP fragments or analogs to bind to a receptor which is recognized rHuAFP can be tested using any standard binding assay known in the art. Biological activity of such fragments and analogs are tested according to methods known in the art (e.g., those described herein).

In general, fragments of rHuAFP are produced according to the techniqu s of polypeptid expression and purification described supra. For example, suitable fragments of rHuAFP can be produced by transformation of a suitable host bacterial cell with part of an HuAFP-encoding cDNA fragment (e.g., the cDNA described above) in a suitable expression vehicle. Alternatively, such fragments can be generated by standard techniques of PCR and cloned into the expression vectors (supra). Fragments can also be produced by chemical synthesis (e.g., by the methods described in *Solid Phase Peptide Synthesis*, 2nd ed., 1984, The Pierce Chemical Co., Rockford, Il). Accordingly, once a fragment of rHuAFP is expressed, it may be isolated by various chromatographic and/or immunological methods known in the art. Lysis and fractionation of rHuAFP-containing cells prior to affinity chromatography may be performed by standard methods. The ability of a candidate rHuAFP fragment to exhibit a biological activity of alpha-fetoprotein is assessed by methods known to those skilled in the art (e.g., those described herein).

As is discussed above, a rHuAFP fragment may also be expressed as a fusion protein with maltose binding protein produced in E. coli. Using the maltose binding protein fusion and purification system (New England Biolabs, Beverly, MA), the cloned human cDNA sequence can be inserted downstream and in frame of the gene encoding maltose binding protein (malE), and the malE fusion protein can then be overexpressed. In the absence of convenient restriction sites in the human cDNA sequence, PCR can be used to introduce restriction sites compatible with the vector at the 5′ and 3′ end of the cDNA fragment to facilitate insertion of the cDNA fragment into the vector. Following expression of the fusion protein, it can be purified by affinity chromatography. For

example, the fusion protein can be purified by virtue of the ability of the maltose binding protein portion of the fusion protein to bind to amylose immobilized on a column.

To facilitate protein purification, the pMalE plasmid contains a factor Xa cleavage site upstream of the site into which the cDNA is inserted into the vector. Thus, the fusion protein purified as described above can then be cleaved with factor Xa to separate the maltose binding protein from a fragment of the recombinant human cDNA gene product. The cleavage products can be subjected to further chromatography to purify rHuAFP from the maltose binding protein. Alternatively, a fragment of rHuAFP may be expressed as a fusion protein containing a polyhistidine tag can be produced. Such an alpha-fetoprotein fusion protein may then be isolated by binding of the polyhistidine tag to an affinity column having a nickel moiety which binds the polyhistidine region with high affinity. The fusion protein may then be eluted by shifting the pH within the affinity column. The rHuAFP can be released from the polyhistidine sequences present in the resultant fusion protein by cleavage of the fusion protein with specific proteases. Recombinant HuAFP fragment expression products (e.g., produced by any of the prokaryotic systems described supra) may be assayed by immunological procedures, such as Western blot, immunoprecipitation analysis of recombinant cell extracts, or immunofluorescence (using, e.g., the methods described in Ausubel et al., *Current Protocols In Molecular Biology*, Greene Publishing Associates and Wiley Interscience (John Wiley & Sons), New York, 1994).

If desired, the purified recombinant gene product or fragment thereof can then be used to raise polyclonal or monoclonal antibodies against the human recombinant

alpha-fetoprotein using well-known methods (see Coligan et al., eds., *Current Protocols in Immunology*, 1992, Greene Publishing Associates and Wiley-Interscience). To generate monoclonal antibodies, a mouse can be immunized with the recombinant protein, and antibody-secreting B cells isolated and immortalized with a non-secretory myeloma cell fusion partner. Hybridomas are then screened for production of recombinant human alpha-fetoprotein (or a fragment or analog thereof)-specific antibodies and cloned to obtain a homogenous cell population which produces monoclonal antibodies.

As used herein, the term "fragment," as applied to a rHuAFP polypeptide, is preferably at least 20 contiguous amino acids, preferably at least 50 contiguous amino acids, more preferably at least 100 contiguous amino acids, and most preferably at least 200 to 400 or more contiguous amino acids in length. Fragments of rHuAFP molecules can be generated by methods known to those skilled in the art, e.g., proteolytic cleavage or expression of recombinant peptides, or may result from normal protein processing (e.g., removal of amino acids from nascent polypeptide that are not required for biological activity).

Recombinant HuAFP fragments of interest include, but are not limited to, Domain I (amino acids 1 (Thr) - 197 (Ser), see Fig. 1, SEQ ID NO: 6), Domain II (amino acids 198 (Ser) - 389 (Ser), see Fig. 1, SEQ ID NO: 7), Domain III (amino acids 390 (Gln) - 590 (Val), see Fig. 1, SEQ ID NO:), Domain I+II (amino acids 1 (Thr) - 389 (Ser), see Fig. 1, SEQ ID NO: 9), Domain II+III (amino acids 198 (Ser) - 590 (Val), see Fig. 1, SEQ ID NO: 10), and rHuAFP Fragment I (amino acids 266 (Met) - 590 (Val), see Fig. 1, SEQ ID NO: 11). Activity of a fragment is evaluated experimentally using conventional techniques and assays, e.g., the assays described herein.

The invention further includes analogs of full-length rHuAFP or fragments thereof. Analogs can differ from rHuAFP by amino acid sequence differences, or by modifications (e.g., post-translational modifications) which do not affect sequence, or by both. Analogs of the invention will generally exhibit at least 80%, more preferably 85%, and most preferably 90% or even 99% amino acid identity with all or part of a rHuAFP amino acid sequence. Modifications (which do not normally alter primary sequence) include in vivo, or in vitro chemical derivatization of polypeptides, e.g., acetylation, or carboxylation; such modifications may occur during polypeptide synthesis or processing or following treatment with isolated modifying enzymes. Analogs can also differ from the naturally occurring rHuAFP by alterations in primary sequence, for example, substitution of one amino acid for another with similar characteristics (e.g., valine for glycine, arginine for lysine, etc.) or by one or more non-conservative amino acid substitutions, deletions, or insertions which do not abolish the polypeptide's biological activity. These include genetic variants, both natural and induced (for example, resulting from random mutagenesis by irradiation or exposure to ethanemethylsulfate or by site-specific mutagenesis as described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Press, 1989, or Ausubel et al., supra)). Also included are cyclized peptide molecules and analogs which contain residues other than L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g., β or γ amino acids, or L-amino acids with non-natural side chains (see e.g., Noren et al., Science 244:182, 1989). Methods for site-specific incorporation of non-natural amino acids into the protein backbone of proteins is described, e.g., in Ellman et al., Science 255:197, 1992.

Also included are chemically synthesized polypeptides or peptides with modifi d peptide bonds (e.g., non-peptide bonds as described in U.S. Pat. No. 4,897,445 and U.S. Pat. No. 5,059,653) or modified side chains to obtain the desired pharmaceutical properties as described herein. Useful mutants and analogs are identified using conventional methods, e.g., those described herein.

Recombinant HuAFP As An Immunosuppressive Agent

Immunosuppressive attributes of rHuAFP (or a fragment or analog thereof) are evaluated by any standard assay for analysis of immunoregulatory activity in vivo or in vitro. As discussed infra, the art provides a number of animal systems for in vivo testing of immunosuppressive characteristics of rHuAFP (or a fragment or analog thereof) on an autoimmune disease, e.g., the nonobese diabetic (NOD) mouse. Furthermore, a wide variety of in vitro systems are also available for testing immunosuppressive aspects of rHuAFP e.g., one such in vitro assay evaluates the inhibition of autoantigen-induced proliferation of T cells in an autologous mixed lymphocyte reaction (AMLR).

The following examples demonstrate that unglycosylated rHuAFP and a fragment of rHuAFP inhibit T cell autoproliferation in response to self antigens. These examples are provided to illustrate, not limit, the invention.

EXPERIMENTAL

MATERIALS AND METHODS

Gel Electrophoresis, Immunoblotting and Purification

The purity and characterization of rHuAFP was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and nondenaturing alkaline PAGE (APAGE) according to standard methods. Gels were subsequently analyzed either by staining with Coomassie

brilliant blue or by transferring electrophoretically separated polypeptides to Immobilon PVDF membranes (Millipore, Mississauga, ON) for immunoblotting analysis. Recombinant HuAFP-monospecific rabbit anti-natural HuAFP polyclonal antibody complexes were identified by alkaline-phosphatase-conjugated goat anti-rabbit IgG and the immunoreactive bands were detected with the BCIP/NBT color development solution (Bio-Rad Laboratories, Mississauga, ON) according to the manufacturer's instructions.

Column chromatography was performed according to standard methods.

Polymerase Chain Reaction (PCR) rHuAFP Fragments

Plasmid constructs encoding fragments of human alpha-fetoprotein were prepared using polymerase chain reaction (PCR) techniques known to those skilled in the art of molecular biology, using oligonucleotide primers designed to amplify specific portions of the human alpha-fetoprotein gene (see e.g., *PCR Technology*, H.A. Erlich, ed., Stockton Press, New York, 1989; *PCR Protocols: A Guide to Methods and Applications*, M.A. Innis, David H. Gelfand, John J. Sninsky, and Thomas J. White, eds., Academic Press, Inc., New York, 1990, and Ausubel et. al., supra).

The following six rHuAFP fragments were prepared to evaluate their biological activity (e.g., according to the -methods disclosed herein):

Domain I	Amino acids 1 (Thr) - 197 (Ser),	(Fig. 1, SEQ ID NO: 6)
Domain II	Amino acids 198 (Ser) - 389 (Ser),	(Fig. 1, SEQ ID NO: 7)
Domain III	Amino acids 390 (Gln) - 590 (Val),	(Fig. 1, SEQ ID NO: 8)
Domain I + II	Amino acids 1 (Thr) - 389 (Ser),	(Fig. 1, SEQ ID NO: 9)
Domain II + III	Amino acids 198 (Ser) - 590 (Val),	(Fig. 1, SEQ ID NO: 10)
rHuAFP Fragment 1	Amino acids 266 (Met) - 590 (Val),	(Fig. 1, SEQ ID NO: 11)

Amino acids sequences were deduced from those shown for human alpha-fetoprotein (1(Thr) - 590 (Val); SEQ ID NO: 5

in Fig. 1. Fragments of rHuAFP designated Domain I, Domain II, Domain III, Domain I+II, Domain II+III and rHuAFP Fragment I were synthesized using standard PCR reaction conditions in 100 μL reactions containing 34 μL H_2O, 10 μL 10X reaction buffer, 20 μL 1 mM dNTP, 2 μL DNA template (HuAFP cloned in pI18), appropriate 5′ and 3′ oligonucleotide primers (10 μL 10 pmol/μL 5′ primer, 10 μL 10 pmol/μL 3′ primer), 1 μL glycerol, 10 μL DMSO, and 1 μL *Pfu* polymerase (Stratagene, LaJolla, CA). Primers used for PCR amplifications were:

DomI25	5′-AAAAAAGGTACCACACTGCATAGAAATGAA-3′	(SEQ ID NO: 14)
DomI3	5′-AAAAAAGGATCCTTAGCTTTCTCTTAATTCTTT-3′	(SEQ ID NO: 15)
DomII5	5-′AAAAAAATCGATATGAGCTTGTTAAATCAACAT-3′	(SEQ ID NO: 16)
DomII3	5′-AAAAAAGGATCCTTAGCTCTCCTGGATGTATTT-3′	(SEQ ID NO: 17)
DomIII5	5′-AAAAAAATCGATATGCAAGCATTGGCAAAGCGA-3′	(SEQ ID NO: 18)
DomIII3	5′-AAAAAAGGATCCTTAAACTCCCAAAGCAGCACG-3′	(SEQ ID NO: 19)
5′rHuAFP Fragment I	5′-AAAAAAATCGATATGTCCTACATATGTTCTCAA-3′	(SEQ ID NO: 20)

Accordingly, primer pairs DomI25 and DomI3, DomII5 and DomII3, DomIII5 and DomIII3, 5′rHuAFP Fragment I and DomIII3, DomI25 and DomII3, and DomII5 and DomIII3 were used to isolate cDNA sequences of Domain I, Domain II, Domain III, rHuAFP Fragment I, Domain I+II, and Domain II+III, respectively, of rHuAFP. Annealing, extension, and denaturation temperatures were 50°C, 72°C, and 94°C, respectively, for 30 cycles. PCR products were purified according to standard methods. Purified PCR products encoding Domain I and Domain I+II were digested individually with KpnI and BamHI and cloned separately into KpnI/BamHI- treated pTrp4. Purified PCR products encoding Domain II, Domain III, Domain II+III, and rHuAFP Fragment I were digested individually with Bsp106I and BamHI and were cloned separately into Bsp106I/BamHI-treated pTrp4. Each plasmid construct was subsequently transformed into competent E. coli cells. Since the expr ssion product will begin with the amino acid

sequence encoded by th translati n start signal methionine, it is xpected that such signal will b removed, or in any event, not affect the bioactivity of the ultimate expression product.

Autologous Mixed Lymphocyte Reactions (AMLR)

Isolation of human peripheral blood mononuclear cells (PBMC), their fractionation into non-T cell populations, and the AMLR, were performed according to standard procedures. Responder T cells were isolated by passing 1.5 x 10^8 PMBC over a commercial Ig-anti-Ig affinity column (Biotek Laboratories) and 2 X 10^5 responder cells were subsequently cultured with 2 x 10^5 autologous ^{137}Cs-irradiated (2500 rads) non-T stimulator cells from a single donor. The medium employed consisted of RPMI-1640 supplemented with 20 mM HEPES (Gibco), 5 x 10^{-5} M 2-mercaptoethanol (BDH, Montreal, QC), 4 mM L-glutamine (Gibco), 100 U/ml penicillin (Gibco) and 100 µg/ml streptomycin sulfate, with the addition of 10% fresh human serum autologous to the responder T cell donor for the AMLR. Varying concentrations of purified rHuAFP, human serum albumin (HSA), anti-HuAFP monoclonal antibodies clone #164 (125 µg/ml final concentration in culture) (Leinco Technologies, St. Louis, MO) were added at the initiation of cultures. AMLR cultures were incubated for 4 to 7 days, at 37°C in 95% air and 5% CO_2. At the indicated intervals, DNA synthesis was assayed by a 6 hour pulse with 1 µCi of ^3H-thymidine (specific activity 56 to 80 Ci/mmole, ICN). The cultures were harvested on a multiple sample harvester (Skatron, Sterling, VA), and the incorporation of ^3H-TdR was measured in a Packard 2500 TR liquid scintillation counter. Results are expressed as mean cpm ± the standard error of the mean of triplicate or quadruplicate cultures.

Peripheral Blood Lymphocyte (PBL) Assays

H parinized blood from n rmal d nors was diluted 1:1 with PBS and human peripheral blood lymphocytes (PBL) were separated from red blood cells by density centrifugation on Ficoll-Hypaque (Sigma, St. Louis, MO). They were washed at least 3 times with PBS and verified for cell viability by the Trypan Blue exclusion method. Human PBL (2.5 x 10^5 cells) were cultured according to standard methods. Results are expressed as the mean cpm thymidine incorporation ± SEM of triplicate cultures.

RESULTS

Expression and Purification

Purity of isolated rHuAFP expressed in E. coli was verified as a single band on Coomassie stained APAGE and SDS-PAGE are shown in Fig. 1A-1B, respectively. Soluble monomeric rHuAFP derived from E. coli was obtained by eluting a protein fraction containing rHuAFP employing Q-sepharose chromatography. Approximately 1 mg of pure rHuAFP per liter of bacterial culture was recovered as a single homogeneous peak by FPLC Mono-Q anion exchange with 220-230 mM NaCl and migrated at approximately 65 kD on SDS-PAGE (Fig. 1B). Recombinant HuAFP exhibits a lower molecular weight on SDS-PAGE than natural HuAFP, since prokaryotic expression systems lack the enzymatic machinery required for glycosylation of proteins. Rechromatographed samples of pure rHuAFP on FPLC and HPLC yielded a single peak as shown in Fig. 1C and Fig. 1D, confirming the purity of the rHuAFP preparation. In addition, N-terminal sequencing data correspond to the expected amino acid sequence at the N-terminus of rHuAFP.

In addition, E. coli containing the expression plasmid encoding rHuAFP were cultured as described above. Fig. 2 (lane D) shows the SDS-PAGE profile of purified rHuAFP Fragment I. N-terminal amino acid sequence analysis showed that rHuAFP Fragment I possessed the

amino acid sequence Ser_{267}-Tyr-Ile-Cys-Ser-Gln-Gln-Asp-Thr_{275} (SEQ ID NO: 13) which corresponds to the expected N-terminal amino acid sequence of rHuAFP Fragment I (see Fig. 1, SEQ ID NO: 11) where the initiating methionine is cleaved intracellularly.

Inhibition of the Autologous Mixed Lymphocyte Reactions (AMLR)

The immunosuppressive activity of rHuAFP was assessed by its ability to suppress human autologous mixed lymphocyte reactions (AMLR). As shown in Fig. 8A, rHuAFP inhibited the proliferative response of autoreactive lymphocytes stimulated by autologous non-T cells, throughout the 4 to 7 day time course measuring autoproliferation. Results from dose-response studies performed at the peak of T cell autoproliferation, as shown in Fig. 8B, demonstrate that the addition of rHuAFP at the initiation of cultures suppressed the AMLR in a dose-dependent manner. Furthermore, parallel viability studies established that the inhibitory activity of rHuAFP on human autoreactive T cells was not due to non-specific cytotoxic effects.

To further substantiate that rHuAFP was the agent responsible for the inhibition of autoproliferating T cells, blocking of rHuAFP-mediated suppression of the AMLR was performed using commercial murine anti-human AFP monoclonal antibodies (MAb). As illustrated in Fig. 5, suppression of proliferating autoreactive T cells by 100 μg/ml of rHuAFP was completely blocked by anti-HuAFP MAb. The addition of 100 μg/ml of HSA did not diminish the AMLR response and the presence of MAb alone in the reaction culture was without any effect.

Recombinant polypeptides produced in prokaryotic expression systems are at risk for contamination with host cell lipopolysaccharide (LPS) during their isolation from bacteria. It has been demonstrated that small

amounts of LPS can antag nize the biological activities of cytokines, thereby impairing the immune responsiveness of macrophages. Accordingly, the effect of endotoxin on various rHuAFP preparations was evaluated by performing AMLR experiments with recombinant protein depleted of endotoxin by passage over Detoxi-gel (Pierce) versus that of rHuAFP which was untreated. Results of these experiments showed that both preparations had equivalent levels of immunosuppressive activity.

As shown in Figure 8A and Figure 8B, the results of this study also demonstrate that rHuAFP suppresses the proliferation of autoreaction T cells with a potency equivalent to glycosylated nHuAFP by eliciting inhibitory effects on autoproliferating T cells throughout the in vitro reactions, with highly significant inhibition being achieved with rHuAFP concentrations ranging from 5 μg/ml to 100 μg/ml. In addition, as shown in Table I and Fig. 3, rHuAFP Fragment I inhibited the proliferative response of autoreactive lymphocytes stimulated by autologous non-T cells at 144 hours. Moreover, as shown in Fig. 3, Domains I and III also inhibited the AMLR.

Table I

Reaction Setup	Thymidine Incorporation (CPM)
T Cells	7118 ± 964
AMLR	83103 ± 6480
AMLR + rHuAFP Fragment I (100 μg/ml)	29692 ± 2963

Inhibition of the Peripheral Blood Lymphocyte Reactions (PBL)

The immunosuppressive activity of 100 μg/ml rHuAFP Fragment I, and E. coli- and baculovirus-derived rHuAFP (described below) was also assessed for the ability to

suppress human peripheral blood lymphocyte reactions (PBL). As shown in Table II, E. coli- and bacul virus-derived rHuAFP and Fragment I (produced as described above) were found to inhibit the proliferative responses of Con A stimulated human peripheral blood lymphocytes.

Table II

	3H-Thymidine Incorporation (CPM±SE)		
No protein	102,353 ± 5,566	91,502 ± 4,333	99,700 ± 4,464
100µg/ml Human Albumin	89,860 ± 5,800	82,924 ± 11,085	94,123 ± 1,633
100µg/ml rHuAFP-E. coli	33,641 ± 3,893	-	-
100µg/ml rHuAFP-Baculovirus	-	31,331 ± 6,303	-
100µg/ml HuAFP Fragment I	-	-	39,019 ± 161

Autoimmune Disease

As is discussed above, autoimmune diseases are characterized by a loss of tolerance to self antigens, causing cells of the immune systems, e.g., T or B cells (or both), to react against self tissue antigens. Autoimmune diseases may involve any organ system, although some are affected more commonly than others. Examples of tissues affected by autoimmune conditions include: the white matter of the brain and spinal cord in multiple sclerosis; the lining of the joints in rheumatoid arthritis; and the insulin secreting β islet cells of the pancreas in insulin-dependent diabetes mellitus. Other forms of autoimmune disease destroy the connections between nerve and muscle in myasthenia gravis or destroy the kidneys and other organs in systemic lupus erythematosus. Examples of other autoimmune diseases include, without limitation, Addison's disease, Crohn's disease, Graves' disease, psoriasis, scleroderma, and ulcerative colitis.

The art provides a wid variety of experim ntal animal systems, transgenic and non-transg nic, for testing therapies for human illness involving autoimmune diseases (see e.g., Paul, W.E., *Fundamental Immunology*, 2nd ed., Raven Press, New York, 1989; and Kandel et al. *Principles of Neural Science*, 3rd ed., Appleton and Lange, Norwalk, CT, 1991; and *Current Protocols In Immunology*, Coligan, J.E., Kruisbeek, A.M., Margulies, D.H., Shevach, E.M., and Strober, eds., Green Publishing Associates (John Wiley & Sons), New York, 1992). Based on the above-described experimental results showing immunosuppressive activity of unglycosylated rHuAFP, it is reasonable to believe that other autoimmune diseases can be treated by administration of such rHuAFP (or a fragment or analog thereof) produced in a prokaryotic system. Accordingly, the invention provides the use of rHuAFP (or a fragment or analog thereof) for treatment (~~i.e., prevention~~ or suppression or amelioration or promotion of remission) of any autoimmune disease.

There now follow examples of animal systems useful for evaluating the efficacy of recombinant human alpha-fetoprotein or an immune cell anti-proliferative fragment or analog thereof in treating autoimmune diseases. These examples are provided for the purpose of illustrating, not limiting, the invention.

Multiple Sclerosis

Multiple sclerosis (MS) is a demyelinating disease involving scattered areas of the white matter of the central nervous system. In MS, myelin basic protein and proteolipid protein are the major targets of an autoimmune response involving T lymphocytes, among other immune system components. Loss of the myelin sheath of nerve cells (demyelination) occurs, resulting in neurological symptoms that culminate in coma or paralysis.

Experimental autoimmune encephalomyelitis (EAE) is a primary model used in the art to examine and assess the effectiveness of therapeutic agents for treating MS. EAE is an inflammatory autoimmune demyelinating disease induced in laboratory animals by immunization with central nervous system tissue. When animals (e.g., mice, rats, guinea pigs, rabbits, monkeys, etc.) are injected with adjuvant, e.g., complete Freund's adjuvant, plus myelin basic protein or proteolipid protein, EAE is induced, which is similar, pathologically to MS (see e.g., Alvord et al., *Experimental Allergic Encephalomyelitis-A Useful Model for Multiple Sclerosis*, Liss, New York, 1984; Swanborg, Meth. Enzymol. 162:413, 1988; and McCarron et al., J. Immunol., 147: 3296, 1991.)

To evaluate rHuAFP or a fragment or analog thereof, EAE is induced in an appropriate laboratory animal, e.g., a mouse or rabbit, according to methods known in the art. To evaluate the compound's immunosuppressive effect on EAE, i.e., its ability to prevent or ameliorate EAE, the compound is administered according to standard methods, e.g., intravenously or intraperitoneal, at an appropriate dosage on a daily basis. Generally, administration is initiated prior to inducing EAE and/or after the clinical appearance of EAE. Control animals receive a placebo, e.g., human serum albumin, similarly administered as for rHuAFP or related molecules. The effect of the test molecules on EAE is monitored according to any standard method. For example, weight loss and muscle paralysis in EAE-induced animals is monitored on a daily basis. If desired, histological inspection (e.g., by using any standard histochemical or immunohistochemical procedure, see e.g., Ausubel et al., *Current Protocols In Molecular Biology*, Greene Publishing Associates (John Wiley & Son), New York, 1994; Bancroft

and Stevens, *Theory and Practice of Histochemical Techniques*, Churchill Livingston , 1982) f brain and spinal cord tissues is performed and tissue samples examined microscopically for evidence of EAE, e.g., evidence of perivascular cellular infiltrates. Comparative studies between treated and control animals are used to determine the relative efficacy of the test molecules in preventing or ameliorating EAE. A molecule which prevents or ameliorates (decreases or suppresses or relieves or promotes remission of) the symptoms of EAE is considered useful in the invention.

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a common chronic illness in which the synovial membrane of multiple joints becomes inflamed, causing damage to cartilage and bone. RA is associated with human lymphocyte antigen (HLA)-DR4 and considered to be an autoimmune disorder involving T cells, see e.g., Sewell et al., Lancet 341: 283, 1993. RA results from a complex interaction of synovial cells with various cellular elements (and their soluble products) that infiltrate from the circulation into the synovial lining of joints. A series of biological events occur which ultimately lead to a lesion which invades and erodes collagen and the cartilage matrix of the joint.

A number of animal models of RA, e.g., the MRL-*lpr/lpr* mouse, are known in the art which develop a form of arthritis resembling the human disease (see e.g., *Fundamental Immunology*, supra). Alternatively, autoimmune collagen arthritis (ACA) and adjuvant arthritis (AA) can be induced in an appropriate animal according to standard methods.

To evaluate rHuAFP or a fragment or analog thereof on immunosuppressive on RA, i.e., the compound's ability to prevent or ameliorate RA, the test molecule is administered to a MRL-*lpr/lpr* mouse according to standard

methods, e.g., intravenously or intraperitoneally, at an appropriate dosage on a daily basis. Generally, administration is initiated prior to the onset of RA and/or after the clinical appearance of RA. Control animals receive a placebo, e.g., human serum albumin, similarly administered as for rHuAFP or related molecules. The effect of the test molecule on RA is monitored according to standard methods. For example, analysis of the cellular component(s) of a synovial joint are monitored on a daily basis. If desired, histological inspection (e.g., by using any standard histochemical or immunohistochemical procedure, see e.g., Ausubel et al., supra; Bancroft and Stevens, supra) of the synovial joint is performed and tissue samples examined microscopically for evidence of RA, e.g., evidence of erosion of collagen and cartilage matrix in a joint. Comparative studies between treated and control animals are used to determine the relative efficacy of the test molecule in preventing or ameliorating RA. A test molecule which prevents or ameliorates (decreases or suppresses or relieves or promotes remission of) the symptoms of RA is considered useful in the invention.

Myasthenia Gravis

Myasthenia gravis (MG) is a disorder of neuromuscular transmission in which there are autoantibodies against acetylcholine receptors of neuromuscular junctions. Antibodies attack the junction, causing weakness and paralysis. Females are afflicted twice as often as males, typically during the third decade of life. Muscular weakness is the predominant feature of the disease. Clinical signs include drooping of the eyelids and double vision. There is an association between MG and hyperthyroidism.

Experimental autoimmune MG (EAMG) has been studied in a variety of animals including rabbits, monkeys, Lewis

rats and inbred strains of mice (see e.g., *Principles of Neural Science*, supra), the symptoms of EAMG resemble the essential characteristics of the human disease. A single injection of acetylcholine receptor, e.g., purified from the electric organs of the eel Torpedo californica, along with adjuvants, causes an acute phase of weakness within 8 to 12 days and then chronic weakness after about 30 days. The response to the eel receptor is T cell dependent. The C57BL/6 strain ($H\text{-}2^B$) is a high responder to Torpedo receptor and highly susceptible to EAMG.

To evaluate rHuAFP or a fragment or analog thereof, EAMG is induced in an appropriate laboratory animal, e.g., the C57BL/6 strain ($H\text{-}2^B$) mouse, according to methods known in the art. To evaluate the compound's immunosuppressive effect on EAMG, i.e., its ability to prevent or ameliorate EAMG, the compound is administered according to standard methods, e.g., intravenously or intraperitoneally, at an appropriate dosage on a daily basis. Generally, administration is initiated prior to inducing EAMG and/or after the clinical appearance of EAMG. Control animals receive a placebo, e.g., human serum albumin, similarly administered as for rHuAFP or related molecules. The effect of the test molecules on EAMG is monitored according to standard methods. For example, nerve stimulation in an electromyographic muscle assay (e.g., according to the methods of Pachner et al., Ann. Neurol. 11:48, 1982) in EAMG-induced animals can be assayed. If desired, histological inspection (e.g., by using any standard histochemical or immunohistochemical procedure, see e.g., Ausubel et al., supra; Bancroft and Stevens, supra) of tissue samples is performed and tissue samples examined microscopically for evidence of EAMG, e.g., evidence of monocyte infiltration and/or autoantibody localization at acetylcholine receptors of neuromuscular junctions. Comparative studies between

treated and control animals are used to determin th relative efficacy of the test molecul s in pr venting or ameliorating EAMG. A molecule which prevents or ameliorates (decreases or suppresses or relieves or promotes remission of) the symptoms of EAMG is considered useful in the invention.

Insulin-Dependent Diabetes Mellitus

Diabetes is a disorder of glucose metabolism. Insulin-dependent diabetes mellitus (IDDM), also known as Type I diabetes, is an autoimmune disease characterized by T-cell mediated destruction of pancreatic β cells in the islets of Langerhans, accompanied by an immune response to a diversity of self peptides leading to hyperglycemia, among other pathological events. IDDM patients depend on exogenous insulin to maintain normal glucose metabolism. Humans at risk for developing IDDM can be identified prior to onset of hyperglycemia by the abnormal occurrence of autoantibodies to insulin, islet cells, glutamic acid carboxylase, as well as other autologous proteins (see e.g., Baekkeskov et al., J. Clin. Invest. 79:926, 1987; Dean et al., Diabetologia 29: 339, 1986; Rossini et al., Annu. Rev. Immunol. 3:289, 1985; Srikanta et al., N. Engl. J. Med. 308:322, 1983). Autoantibody patterns, in general, are predictive for the eventual disease progression and/or risk for developing the disease (see e.g., Keller et al., Lancet 341:927, 1993).

Examples of animal models which spontaneously develop IDDM resembling the human disease include the Bio-Breeding (BB) rat and nonobese diabetic (NOD) mouse. Diabetes is also experimentally induced by streptozotocin.

The BB rat spontaneously develops a disease similar to IDDM, with insulitis (infiltration of mononuclear cells into the pancreatic islets) and

aut antibodies against self cells and insulin (see .g., Baekkeskov et al., J. Clin. Invest. 79:926, 1987; R ssini et al, supra; Nakhooda et al., Diabetes 26: 100, 1977; Dean et al., Clin. Exp. Immunol. 69: 308, 1987).

NOD mice typically develop insulitis between 5 and 8 weeks of age, and by 7 months 70% of the females and 40% of the males become diabetic. T cells transferred from diabetic mice to young nondiabetic NOD mice induce diabetes within 2 to 3 weeks (see e.g., Bendelac et al., J. Exp. Med. 166:823, 1987). NOD mice usually die within 1 to 2 months after the onset of diabetes unless they receive insulin therapy.

Chemically induced diabetes is accomplished using multiple injections of small doses of streptozotocin, a drug toxic for pancreatic β cells, which causes severe insulitis and diabetes (see e.g., Kikutani et al., Adv. Immunol. 51:285, 1992).

Accordingly, the art provides a variety animal models resembling human IDDM which can be used to examine and assess approaches for the prevention or amelioration of diabetes involving rHuAFP (or a fragment or analog thereof).

To evaluate the immunosuppressive effect of rHuAFP or a fragment or analog thereof on the development of diabetes mouse, i.e., the compound's ability to treat or prevent insulitis and diabetes, the test compound is administered to an appropriate test animal, e.g, a NOD mouse, according to standard methods, e.g., intravenously or intraperitoneally, at an appropriate dosage on a daily basis. Generally, administration is initiated prior to the onset of insulitis and diabetes and/or after the clinical appearance of diabetic characteristics. Control animals receive a placebo, e.g., human serum albumin, similarly administered as for rHuAFP or related molecules. The effect of test molecules on insulitis and

diab tes is monitored according to standard methods. For example, weight l ss, keton body formati n, and blood glucose concentration is monitored on a daily basis. If desired, histological inspection (e.g., by using any standard histochemical or immunohistochemical procedure, see e.g., Ausubel et al., supra; Bancroft and Stevens, supra) of pancreatic islet cells is performed and tissue samples examined microscopically for evidence of insulitis and β cell destruction. Comparative studies between treated and control animals are used to determine the relative efficacy of the test molecules in preventing or ameliorating the diabetic condition. A molecule which prevents or ameliorates (decreases or suppresses or relieves or promotes remission of) the symptoms of diabetes, e.g., IDDM, is considered useful in the invention.

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a severe systemic autoimmune disease. About 90% of patients with this disease are young women. This marked preponderance of females is not seen before puberty or after menopause. The illness generally begins in young adulthood when a characteristic skin rash appears over cheekbones and forehead. Hair loss is common, as is severe kidney damage, arthritis, accumulation of fluid around the heart and inflammation of the lining of the lungs. In nearly half of the patients the blood vessels of the brain also become inflamed, leading to paralysis and convulsions. The activity of the disease, like other autoimmune diseases, can fluctuate: long quiescent periods of good health can terminate abruptly and inexplicably with the onset of a new attack. A large number of different autoantibodies are known to occur in SLE, e.g., autoantibodies against DNA, RNA and histones (see, e.g., *Fundamental Immunology*, supra)

A number of animal models of human SLE, e.g., inbred mouse strains including NZB mice and their F_1 hybrids, MRL mice, and BXSB mice, are known in the art (see e.g., Bielschowsky et al. Proc. Univ. Otago Med. Sch. 37:9, 1959; Braverman et al., J. Invest. Derm. 50: 483, 1968; Howie et al. Adv. Immunol. 9:215, 1968; *Genetic Control of Autoimmune Disease*, Rose, M., Bigazzi, P.E., and Warner, N.L. eds., Elsevier, Amsterdam, 1979; and *Current Protocols In Immunology*, supra). For example, the NZBxNZW F_1 mouse is an excellent model of human SLE, female mice develop high levels of anti-doubl - and single-stranded DNA autoantibodies, other anti-nuclear antibodies, and renal disease; death usually occurs at approximately 8 months (see e.g., Theofilopoulos et al., Adv. Immunol. 37:269, 1985).

To evaluate the immunosuppressive effect of rHuAFP or a fragment or analog thereof on SLE, i.e., the compound's ability of rHuAFP to prevent or ameliorate SLE, test compounds are administered to an appropriate animal, e.g., the NZBxNZW F_1 mouse, according to standard methods, e.g., intravenously or intraperitoneally, at an appropriate dosage on a daily basis. Generally, administration is initiated prior to the onset of SLE and/or after the clinical appearance of SLE. Control animals receive a placebo, e.g., human serum albumin, similarly administered as for rHuAFP or related molecules. The effect of the test compound on SLE is monitored according to standard methods. For example, analysis of autoantibodies, e.g., anti-DNA antibodies can be monitored. If desired, histological inspection (e.g., by using any standard histochemical or immunohistochemical procedure, see e.g., Ausubel et al., supra; Bancroft and Stevens, supra) of kidney tissue is performed and tissue samples examined microscopically for evidence of SLE, e.g., evidence of lupus nephritis.

Comparative studies between treated and control animals are used to determine the relative efficacy of the test compounds in preventing or ameliorating SLE. A molecule which prevents or ameliorates (decreases or suppresses or relieves or promotes remission of) the symptoms of SLE is considered useful in the invention.

Therapeutic Administration

As demonstrated above, recombinant alpha-fetoprotein, e.g., rHuAFP (or a fragment or analog thereof) is effective in inhibiting proliferation of autoimmune cells and accordingly is useful for the prevention or amelioration of autoimmune diseases including, but not limited to, multiple sclerosis, rheumatoid arthritis, diabetes mellitus, systemic lupus erythematosus, and myasthenia gravis. Accordingly, recombinant human alpha-fetoprotein (or a fragment or analog thereof) can be formulated according to known methods to prepare pharmaceutically useful compositions. Recombinant alpha-fetoprotein, e.g., rHuAFP (or a fragment or analog thereof), is preferably administered to the patient in an amount which is effective in preventing or ameliorating the symptoms of an autoimmune disease. Generally, a dosage of 0.1 ng/kg to 10 g/kg body is adequate. If desired, administration is performed on a daily basis. Because there are no known adverse side effects related to recombinant human alpha-fetoprotein, it is believed that relatively high dosages can be safely administered. For example, treatment of human patients will be carried out using a therapeutically effective amount of rHuAFP (or a fragment or analog thereof) in a physiologically acceptable carrier. Suitable carriers and their formulation are described for example in Remington's Pharmaceutical Sciences by E.W. Martin. The amount of rHuAFP to be administered will vary depending upon the manner of administration, the age and body

weight of the patient, and with the type of disease, and size of the patient predisposed to or suffering from the disease. Preferable routes of administration include, for example, subcutaneous, intravenous, intramuscular, or intradermal injections which provide continuous, sustained levels of the drug in the patient. In other preferred routes of administration, rHuAFP can be given to a patient by injection or implantation of a slow release preparation, for example, in a slowly dissociating polymeric or crystalline form; this sort of sustained administration can follow an initial delivery of the drug by more conventional routes (for example, those described above). Alternatively, rHuAFP can be administered using an infusion pump (e.g., an external or implantable infusion pump), thus allowing a precise degree of control over the rate of drug release, or through installation of rHuAFP in the nasal passages in a similar fashion to that used to promote absorption of insulin. As an alternative to nasal transmucosal absorption, rHuAFP can be delivered by aerosol deposition of the powder or solution into the lungs.

Furthermore, the method(s) of the invention can also employ combination therapy in which rHuAFP is administered either simultaneously or sequentially with a therapeutic agent such as a general or specific tolerizing agent, e.g., an anti-idiotypic agent (e.g., a monoclonal) or a therapeutic vaccine or an oral agent (e.g., insulin, collagen or myelin basic protein) or a cytokine (e.g., Il-15) or an interferon (α-interferon) or an immunosuppressive agent. Preferably, an immunosuppressive agent is administered in an effective dose which is lower than the standard dose when the immunosuppressive agent is used by itself. Preferred immunosuppressive agents are cyclosporine, FK-506, steroids, azathioprine, or 15-deoxyspergualin.

Treatment is started generally with the diagnosis or suspicion of an autoimmun disease and is generally repeated on a daily basis. Protection or prevention from the development (or progression or exacerbation) of an autoimmune disease is also achieved by administration of rHuAFP prior to the onset of the disease. If desired, the efficacy of the treatment or protection regimens is assessed with the methods of monitoring or diagnosing patients for autoimmune disease.

Recombinant Human AFP in the Treatment and Diagnosis of Cancer

CYTOTOXIC AGENTS

A hybrid cytotoxin of rHuAFP is prepared by conjugating a full-length rHuAFP or a fragment or analog thereof to any number of known toxic entities using conventional techniques. Such toxins are useful for inhibiting the development of a neoplasm (as described infra). Useful cytotoxins are preferably significantly cytotoxic only when present intracellularly and are substantially excluded from any given cell in the absence of a targeting domain. As described below, peptide toxins fulfill both of these criteria and are readily incorporated into hybrid molecules. If desired, a mixed cytotoxin (i.e., a cytotoxin composed of all or part of two or more toxins) can also be used. Several useful toxins are described in more detail below.

Toxin molecules useful in the method of the invention are preferably toxins, such as peptide toxins, which are significantly cytotoxic only when present intracellularly. Of course, under these circumstances the molecule must be able to enter a cell bearing the targeted receptor. This ability depends on the nature of the molecule and the nature of the cell receptor. For example, cell receptors which naturally allow uptake of a ligand are likely to provide a means for a molecule which

includ s a toxin to enter a cell bearing that receptor. As is discussed below, the p ptide toxin useful in the methods of the invention is fused to a rHuAFP (or fragment or analog thereof) binding domain by producing a recombinant DNA molecule which encodes a hybrid protein molecule.

Many peptide toxins have a generalized eukaryotic receptor binding domain; in these instances the toxin must be modified to prevent intoxication of non-receptor bearing cells. Any such modifications must be made in a manner which preserves the cytotoxic functions of the molecule (see U.S. Department of Health and Human Services, U.S. Serial No. 401,412). Potentially useful toxins include, but are not limited to: cholera toxin, ricin, O-Shiga-like toxin (SLT-I, SLT-II, SLT II_v), LT toxin, C3 toxin, Shiga toxin, pertussis toxin, tetanus toxin, Pseudomonas exotoxin, saporin, modeccin, and gelanin.

The cytotoxic portion of some molecules useful in the invention, if desired, can be provided by a mixed toxin molecule. A mixed toxin molecule is a molecule derived from two different polypeptide toxins. Generally, as discussed above, polypeptide toxins have, in addition to the domain responsible for generalized eukaryotic cell binding, an enzymatically active domain and a translocation domain. The binding and translocation domains are required for cell recognition and toxin entry respectively. The enzymatically active domain is the domain responsible for cytotoxic activity once the molecule is inside a cell.

Naturally-occurring proteins which are known to have a translocation domain include diphtheria toxin, Pseudomonas exotoxin A, and possibly other peptide toxins. The translocation domains of diphtheria toxin and Pseudomonas exotoxin A are well characterized (see,

.g., Hoch et al., Proc. Natl. Acad. Sci. USA 82:1692, 1985; Col mbatti t al., J. Bi l. Chem. 261:3030, 1986; and Deleers et al., FEBS Lett. 160:82, 1983), and the existence and location of such a domain in other molecules may be determined by methods such as those employed by Hwang et al. Cell 48:129, 1987); and Gray et al. Proc. Natl. Acad. Sci. USA 81:2645, 1984).

For example, one useful rHuAFP/mixed toxin hybrid molecule is formed by fusing the enzymatically active A subunit of E. coli Shiga-like toxin (see, e.g., Calderwood et al., Proc. Natl. Acad. Sci. USA 84:4364, 1987) to the translocation domain (amino acid residues 202 through 460) of diphtheria toxin, and to rHuAFP. The rHuAFP portion of the three-part hybrid causes the molecule to attach specifically to cells bearing receptors which is recognized by rHuAFP, and the diphtheria toxin translocation portion acts to insert the enzymatically active A subunit of the Shiga-like toxin into the targeted cell. The enzymatically active portion of Shiga-like toxin, like diphtheria toxin, acts on the protein synthesis machinery of the cell to prevent protein synthesis, thus killing the cell.

Functional components of the hybrid cytotoxins of the invention are linked together via a non-covalent or covalent bond, or both. Non-covalent interactions can be ionic, hydrophobic, or hydrophilic, such as interactions involved in a leucine-zipper or antibody-protein G interaction (see, e.g., Derrick et al., Nature 359:752, 1992). An example of a covalent linkage is a disulfide bond.

A hybrid cytotoxin is prepared by chemically conjugating rHuAFP (or fragment or analog) to a any number of known toxic entities, e.g., those described above. Such reactions are carried out by standard techniques known to those skilled in the art. A typical

way of conjugating a protein to a pr tein toxin (including, .g., bacterial toxins such as diphth ria toxin or Pseudomonas exotoxin A, or plant toxins such as ricin) is by crosslinking through a disulfide bond (see, e.g., Chang et al., J. Biol. Chem. 252:1515, 1977) or a heterobifunctional molecule (see, e.g., Cawley et al. Cell 22:563, 1980). See also Stevens et al., U.S. Pat. No. 4,894,227.

Alternatively, the hybrid cytotoxin is prepared by expression of a hybrid DNA engineered to encode both the rHuAFP (or a fragment or analog thereof) and the toxin (or a toxic portion thereof), using technology available to those of ordinary skill in the art of making such hybrids (see, e.g., Murphy, U.S. Pat. No. 4,675,382, and Chadhary et al., Proc. Natl. Acad. Sci. USA 84:4538, 1987). For example, a recombinant fusion protein of rHuAFP and a cytotoxic agent is made according to methods known in the art (see, e.g., Murphy supra and Huston et al., Meth. Enzymol. 203:46, 1991). If the hybrid cytotoxin is produced by expression of a fused gene, a peptide bond serves as the link between the cytotoxic agent and the targeting ligand. Another method useful for conjugating a protein or polypeptide to a protein toxin employs the polymer, monomethoxy-polyethylene glycol (mPEG), as described in Maiti et al., Int. J. Cancer Suppl. 3:17, 1988.

If desired, following its synthesis, the hybrid cytotoxin is affinity purified according to standard methods using antibodies against the targeting portion of the molecule, e.g., antibodies against human alpha-fetoprotein. Similarly, antibodies directed against the cytotoxic agent are also useful for purifying the hybrid cytotoxin molecule by standard immunological techniques. The resulting hybrid cytotoxin is then formulated for use as an agent against unwanted cells, e.g. cancer cells,

following procedures standard in the field of pharmacology.

Molecules of the invention can be screened for the ability to decrease viability of cells bearing the targeted receptor by means of assays known in the art, e.g., those methods described herein.

Because hybrid cytotoxins of the invention are potent cytotoxic agents for cells bearing the a receptor which is recognized by rHuAFP, rHuAFP is useful in the treatment of diseases involving unwanted alpha-fetoprotein receptor-positive cells, e.g., cancer cells.

DIAGNOSTIC AGENTS

Recombinant rHuAFP or a fragment or analog thereof can be attached to a detectable label to produce an agent useful for detecting and localizing a neoplasm in vivo, in situ, or in vitro . Methods for attaching such labels to proteins are known in the art. For example, a detectable label is attached by chemical conjugation, but where the label is a polypeptide, it could alternatively be attached by genetic engineering techniques.

Detectable labels are generally selected from a variety of such labels known in the art, but are normally radioisotopes, flurophores, enzymes (e.g., horseradish peroxidase), or other moieties or compounds which emit a detectable signal (e.g., radioactivity, fluorescence, color) or emit a detectable signal after exposure of the label to its substrate. Various detectable label/substrate pairs (e.g., horseradish peroxidase/diaminobenzidine, avidin/streptavidin, luciferase/luciferin, β-galactosidase/X-gal(5-Bromo-4-Chloro-3-Indoyl-D-Galactopyranoside), and methods for labelling proteins for such detection purposes are known in the art. The usefulness of such an agent can be assayed, for example, by implanting a tumor cell line, e.g, MCF-7, into a host, e.g., a mouse, and determining

whether the agent of the invention detectably labels the tumor produced by such implanted cells, e.g., by radioimaging using scintigraphy. Such an agent can also be used to assay for the presence of any unwanted cell bearing an alpha-fetoprotein receptor, e.g., by using Western blot analysis or histochemical staining of a tissue sample, according to known methods.

Recombinant HuAFP as an Anti-Cancer Agent

Anti-cancer agents of the invention (e.g., rHuAFP or a fragment or analog thereof; or a hybrid cytotoxin of rHuAFP) are useful for inhibiting a neoplasm, e.g., breast or prostate carcinomas. Those skilled in the art will understand that any number of methods, both in vitro and in vivo, are used to determine the efficacy of anti-cancer agents useful in the methods of the invention. For example, the reduction of tumor growth can be monitored in a mouse or rat growing a prostate cancer (e.g., tumor xenografts of LNCaP-androgen receptor-positive human prostate cancer cell line) following the administration of the test compound. In a working example, a human tumor cell lines (e.g., cell lines such as MCF-7(ATCC HTB 22), T-47D (ATCC HTB 19), NIH:OV-CAR-3 (ATCC HTB 161), LnCaP.FGC (ATCC CRL 1740), and Du-145 (ATCC HTB 81) growing in culture is released from monolayer by trypsinization, diluted into single-cell suspension and then solidified by centrifugation into a pellet which is subsequently exposed to 15 µl fibrinogen (50 mg/ml) and 10 µl thrombin (50 units/ml) for 30 minutes at 37°C. Fibrin clots containing tumor are then cut into pieces approximately 1.5 mm in diameter. Each piece of tumor is subsequently implanted under the kidney capsule of a mouse according to standard methods. If desired, mice can be immunosuppressed by daily subcutaneous (s.c.) injection of 60 mg/kg cyclosporine A (Sandimmune IV)

beginning immediately prior to tumor implantation according to conventional methods. If necessary, estrogen and androgen supplementation of mice is achieved by standard methods, e.g, implantation of silastic tubing containing estradiol or by injection of testosterone propionate. Typically, hormone supplementation is commenced on the day of tumor implantation. Generally, administration of the test molecule is initiated prior to tumor implantation and/or after tumor implantation. Control animals receive a placebo, e.g., human serum albumin or diluent, similarly administered as for rHuAFP or related molecules. The effect of the test molecule on tumor growth is monitored according to any standard method. For example, tumor growth is monitored by weekly measurement of tumor size by laparotomy using a dissecting microscope equipped with an ocular micrometer. A molecule shown experimentally to halt or reduce or inhibit the growth of such implanted tumors is considered useful in the invention.

Toxicity of test compounds towards cells bearing receptors that are recognized by rHuAFP can be tested in vitro according to any standard protocol. For example, a cultured cancer cell line, e.g., MCF-7 estrogen-receptor-positive human breast cancer cell line, is maintained in plastic tissue culture flasks (Costar) in DMEM with penicillin (100 U/ml), streptomycin (100 μg/ml), 5% fetal calf serum, insulin (10 ng/ml), L-glutamine (2 mM) and non-essential amino acids (1%). Cells are seeded in 96-well V-bottomed plates (Linbro-Flow Laboratories, McLean, VA) at a concentration of 1 x 10^5 per well in complete medium. Putative toxins are added to varying concentrations (10^{-12}M to 10^{-6}M) and the cultures are incubated for 18 hrs. at 37°C in a 5% CO_2 atmosphere. Following incubation, the plates are centrifuged for 5 min. at 170 x g, and the medium removed

and r placed with 100 μl leucine-free medium (MEM, Gibco) containing 8 μCi/ml (^{3}H-leucine; New England Nuclear, Boston, MA). After an additional 90 min. at 37°C, the plates are centrifuged for 5 min. at 170 x g, the medium is removed, and the cells are collected on glass fiber filters using a cell harvester (Skatron, Sterling, VA). Filters are washed, dried, and counted according to standard methods. Cells cultured with medium alone serv as the control. A test compound which reduces or halts or inhibits cell growth compared to untreated control cells, is detected as an indication of toxicity and is considered useful in the invention.

Evaluation of whether a test compound confers protection against the development of a neoplasm (e.g., breast or prostate cancers) generally involves using an animal known to develop a neoplasm (e.g., the transgenic mouse described in U.S. Pat. No. 4,736,866). An appropriate animal is treated with the test compound according to standard methods, and a reduced incidence of neoplasm development, compared to untreated control animals, is detected as an indication of protection.

As is discussed below, I have discovered that unglycosylated rHuAFP produced in a prokaryotic expression system is effective in treating cancer. For example, rHuAFP has been found to be a potent inhibitor of breast carcinoma growth in vitro.

The experimental examples described below demonstrate the efficacy of rHuAFP as an anti-cancer agent. These examples are provided to illustrate, not limit, the invention.

EXPERIMENTAL

MATERIALS AND METHODS

Culture Media and Tumor Cells

Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, fetal calf serum, glutamine, non-essential amino

acids and p nicillin-streptomycin mixture were btained from GIBCO (BRL). Donor calf serum was obtained from Hyclone, Logan, UT, and porcine insulin was obtained from Squibb, Inc., Princeton, NJ.

The MCF-7 estrogen-receptor-positive human breast cancer cell line was obtained from Dr. Alberto C. Baldi, Institute of Experimental Biology and Medicine, Buenos Aires, Argentina. Stock cultures were maintained in plastic tissue culture flasks (Costar) in DMEM with penicillin (100 U/ml), streptomycin (100 µg/ml), 5% fetal calf serum, insulin (10 ng/ml), L-glutamine (2 mM) and non-essential amino acids (1%).

Estrogen-Stimulated Post-confluent Growth of MCF-7 Cells in Culture.

This assay is based on the finding that MCF-7 cells in estrogen-containing medium grow past confluence and accumulate into foci; but, in the absence of estrogen, cell proliferation stops after the cultures establish cell-cell contact, and no foci are formed (see, e.g., Gierthy et al., Breast Cancer Res. Treat. 12:227, 1988). 1 x 10^7 MCF-7 breast cancer cells were seeded in 16-mm wells contained in 24-well tissue culture plates. Culture medium was phenol red-free DMEM supplemented with 5% donor calf serum (prescreened for absence of detectable estrogens), L-glutamine (2 mM), non-essential amino acids (1X, GIBCO), insulin (10 ng/ml), penicillin-streptomycin (1X, GIBCO) and estradiol diluted to a final concentration of 1.8 X 10^{-9} M. Cultures were refed at 24 hr and every 4 days thereafter with 2 ml of culture medium containing rHuAFP and human serum albumin to yield a final protein concentration of 100 µg/ml per well. Cells reached confluence within 5 days, and a substantial number of foci were apparent within 10 days in wells containing estrogen alone. Cells were fixed with buffered formalin and stained with 1% Rhodamine B. The

stained foci were quantitated using an Artek 870 Macro-Micro Automat d C lony Counter. Data are pres nted as mean number of foci per treatment group.

RESULTS

Activity of rHuAFP Against MCF-7 Breast Cancer Cells

The results shown in Fig. 9 demonstrate that rHuAFP inhibits estrogen-stimulated postconfluent growth of MCF-7 breast cancer cells in vitro. Control experiments using human albumin or no protein had no effect on MCF-7 foci formation. These data indicate that rHuAFP has a direct inhibitory effect on the growth of carcinoma cell cultures.

Therapeutic Administration

As demonstrated above, rHuAFP is effective in inhibiting a neoplasm, e.g., a breast cell carcinoma. Accordingly, compounds of the invention can be formulated according to known methods to prepare pharmaceutically useful compositions. Treatment of human patients will be carried out using a therapeutically effective amount of an anti-cancer agent of rHuAFP in a physiologically acceptable carrier. Suitable carriers and their formulation are described for example in Remington's Pharmaceutical Sciences by E.W. Martin. The amount of the anti-cancer agent to be administered varies depending upon the manner of administration, the age and body weight of the patient, and with the type of disease, extensiveness of the disease, and size of the patient suffering from the disease. Generally amounts will be in the range of those used for other agents used in the treatment of cancer, although in certain instances lower amounts will be needed because of the increased specificity of the compound. For example, rHuAFP is administered systemically, as described below, at a

dosag that inhibits malignant cell proliferation, typically in th range of 0.1 ng - 10 g/kg body weight.

Furthermore, the method of the invention can also employ combination therapy in which rHuAFP is administered either simultaneously or sequentially with a chemotherapeutic agent. Typically, a chemotherapeutic agent is administered according to standard methods or,alternatively, in a dose which is lower than the standard dose when the chemotherapeutic agent is used by itself. Examples of chemotherapeutic agents include, without limitation, mechlorethamine, cyclophosphamide, ifosfamide, L-sarcolysin, chlorambucil, hexamethylmelamine, thiotepa, busulfan, carmustine, lomustine, semustine, streptozocin, dacarbazine, methotrexate, fluorouracil, cytarabine, mercaptopurine, thioguanine, pentostatin, vinblastine, vincristine, etoposide, teniposide, actinomycin D, daunomycin, doxorubicin, bleomycin, plicamycin, mitomycin, cisplatin, mitoxantrone, hydroxyurea, procarbozine, mitotane, aminoglutethimide, prednisone, hydroxyprogesterone, diethylstilbestrol, tamoxifen, flutamide, or leuprolide.

Treatment is started generally with the diagnosis or suspicion of a neoplasm and is generally repeated on a daily basis. Protection from the development of neoplasm is also achieved by administration of rHuAFP on a daily basis. If desired, the efficacy of the treatment or protection regimens is assessed with the methods of monitoring or diagnosing patients for cancer.

Furthermore, the compounds of the invention can also be used to treat mammals to destroy any unwanted cells bearing alpha-fetoprotein receptors associated with a pathological condition. The method(s) of the invention can also be used to treat non-human mammals, for example, domestic pets, or livestock. As described below, the

anti-cancer agents of the inventi n can be administer d systemically or locally.

Systemic Administration

For use as an anti-cancer agent, the compounds of the invention can be administered systemically, for example, formulated in a pharmaceutically-acceptable buffer such as physiological saline. Preferable routes of administration include, for example, subcutaneous, intravenous, interperitoneally, intramuscular, or intradermal injections which provide continuous, sustained levels of the drug in the patient. In other preferred routes of administration, the compounds of the invention can be given to a patient by injection of a slow release preparation, for example, in a slowly dissociating polymeric or crystalline form; this sort of sustained administration can follow an initial delivery of the drug by more conventional routes (for example, those described above). Alternatively, the compounds can be administered using an infusion pump, thus allowing a precise degree of control over the rate of drug release, or through installation of the compounds in the nasal passages in a similar fashion to that used to promote absorption of insulin. As an alternative to nasal transmucosal absorption, the compounds can be delivered by aerosol deposition of the powder or solution into the lungs.

Local Administration

The anti-cancer agents of the invention also can be administered locally to treat cancer. Since the desired action of the agent is generally upon a circumscribed mass of tissue, for example a tumor, delivery of the drug by means which result in high local concentrations in the vicinity of the tumor is especially desirable.

R combinant HuAFP as a Diagnostic Agent

R combinant HuAFP (r fragment r analog thereof) linked to a detectable label finds diagnostic use in the detection or monitoring or assaying for the presence of a neoplasm (e.g., breast or prostate cancers).

For example, in vivo studies can be conducted on human patients to determine the presence of a neoplasm using a detectably labelled rHuAFP (e.g., Tc-99m-labelled rHuAFP). In general, the detectably labelled rHuAFP is administered intravenously and imaging can be performed using scanners by methods known to those skilled in the art, e.g., by radioimaging using scintigraphy.

In another working example, a neoplasm or any cell bearing a receptor which is recognized by rHuAFP may be detected in a tissue sample, e.g., a biopsy, a bodily fluid, by using rHuAFP (or fragment or analog thereof) linked to a detectable label. After determining that a patient should be tested for the presence of such cells, a tissue sample, a biopsy, or a sample of bodily fluid, preferably lymph, blood, serum, or urine, is collected from the patient. Accordingly, the subcellular location or presence of a receptor which is recognized by rHuAFP is determined either in situ or in vitro using fractionated cells by any standard biochemical or histochemical procedure (see e.g., Ausubel et al., supra; Bancroft and Stevens, Theory and Practice of Histological Techniques, Churchill Livingstone, 1982). Appropriate control samples for the assay include a tissue sample or a bodily fluid collected from individuals who do not have cells bearing alpha-fetoprotein (negative control), or samples which contain a known, predetermined amount of alpha-fetoprotein receptor (positive control).

The diagnostic assay may be performed in solution or may use a solid (insoluble) support (e.g. polystyrene, nitrocellul se, or beads) or in a tissue sample prepared

for histological examination, using any standard methods. For example, to d termine whether the patient from whom the test sample was collected has cells bearing receptors which is recognized by rHuAFP, the level of binding of the detectably labelled rHuAFP in the test sample is compared to the level of binding in the negative and/or positive control samples. A level of binding in the test sample greater than the level of binding in the negative control sample, or at least equal to the level of binding in the positive control sample, indicates that the patient has cells bearing alpha-fetoprotein receptors.

Materials for performing the diagnostic assays according to the methods of the invention may be provided as a kit having instructions for use. In general, the kit is composed in part of a rHuAFP (or fragment or analog thereof). This kit may further include a second reagent, e.g., a detectable label, which is used to label rHuAFP (or a fragment or analog thereof). The kits exemplified above are useful in, for example, detecting the presence of a tumor in a sample of human tissue in vitro, or for in vivo examination purposes.

The experimental examples described below demonstrate the efficacy of rHuAFP diagnosing a neoplasm. These examples are provided to illustrate, not limit, the invention.

EXPERIMENTAL

MATERIALS AND METHODS

Animals

MCF-7 human breast cancer cells implanted in the lateral thorax region of CB-17 SCID mice were grown to a size of 1 cm diameter (approx. 5 gm) under estrogen stimulation according to methods known in the art.

Technetium Labelling

99mTc-recombinant labelled alpha-fetoprotein was prepared from an AFP aliquot mixed with 0.5 ml 0.9%

sodium chloride injection soluti n (Baxter H althcare Corporation, Deerfield, IL). The solution is added to an UltraTag RBC Reaction Vial (Mallinckrodt Medical Inc., St. Louis, MO 63134 Lot No. 0683040), containing stannous chloride dihydrate, sodium citrate dihydrate, and dextrose anhydrous, in a lyophilized form stored under argon. The contents of the vial are mixed by gentle swirling, and incubated at room temperature for 5 minutes. At the completion of the incubation, 0.8-1.2 Gbq Technetium 99mTc Sodium Pertechnetate Injection is added (99mTc Generator Mallincrokt Medical, Inc. St. Louis, MO) in a volume of 1-2 ml. The contents of the vial are mixed by gentle swirling and incubated for 15 minutes. Dose aliquots were assayed at 0, 3, and 6 hours after preparation. Thin-layer chromatography performed on preparations using ITLC-SG (Gelman Instrument Co., Ann Arbor, MI) with 0.9% NaCl showed 95-99% of the 99Tc was bound to the recombinant-alpha-fetoprotein.

Imaging

Experimental animals are sedated with Medafane. A 24 gauge, 3/4 inch catheter (Surflo IV catheter, Terumo Medical) is then secured in a lateral tail vein. The animal is then further anesthetized with a slow infusion of 20-25 mg/kg body weight of pentobarbital intravenously. Anesthesia is maintained for restraint as required with injections of 5 mg of additional pentobarbital.

Isotope biodistribution data is collected using a Elscint Dymax 409 gamma camera. This data is subsequently analyzed by a computer (Siemans Gammasonics Microdelta). Animals are imaged in triples, being placed in the dorsal recumbent position on a thin polyethylene panel. To eliminate motion during imaging, the animals are restrained as necessary, on these panels by strips of tape over their extremities so as not to restrict

r spiration. Dynamic imag s obtained over 60 minutes are used to det rmine the biodistribution of the labeled protein. Typically, twelve sequential, five minute images are obtained with low energy general purpose collimation, and 1.5 hardware zoom into the computer matrices having 128 by 128 picture elements.
Study animals are typically injected with 37MBq of Tc-99m labelled protein.

RESULTS

Tracer Biodistribution and Kinetics

Following administration of 37MBq (approx. 4-6 μg Tc-99m recombinant human alpha-fetoprotein) in the tail vein, tracer biodistribution kinetics were measured during the initial hour after injection and at 24 hours. Tissue uptake kinetics were measured in % injected activity/per 100 ROI (Region of Interest) pixels (%IA). During the first hour there is rapid renal clearance, mild localization in the liver and little evident activity in other tissues. At 1 hour, tumor uptake was (mean ± SEM: 1.9 ± 0.3%IA) and the tumor to heart (T/H) region ratio was 0.84 ± 0.23. By 24 hours, tumor uptake was (0.8+/-0.1%IA) and T/A and tumor to background (T/B upper chest) region ratios were 1.43 ± 0.41 and 2.66 ± 0.54, respectively. Studies comparing 99mTc-labelled rHuAFP to 99mTc-labelled human serum albumin (used as a non-specific protein control) repeated in the same animals showed that T/B image ROI activity ration was 2.7 and 5.8 for 99mTc-labelled rHuAFP at 1 and 24 hr, respectively and at 24 hours was 40% greater for 99mTc-labelled rHuAFP compared to Tc-99m human serum albumin. These results show that rHuAFP can be labelled with Tc-99m and that this labelled agent has low non-specific tissue uptake and rapid renal clearance from the blood. Localization in human breast cancer xenografts is initially rapid, increases with time, and is due to

specific tum r uptake. Th se results demonstrate that rHuAFP labelled with Tc-99m is useful as a diagnostic agent for breast carcinoma.

Diagnostic Administration

As discussed above, rHuAFP (or fragment or analog thereof) linked to a detectable label finds diagnostic use in the detection or monitoring or assaying of a neoplasm (e.g., a breast cell carcinoma). Accordingly, patients who present with the classical symptoms of cancer, e.g., breast cancer or prostate cancer, or have a medical history which indicates susceptibility to such cancer may be tested with the methods of the invention. Other appropriate patients for such testing include those who have a family history of breast or prostate carcinomas. Patients who are receiving drugs or have been exposed to toxins implicated in the induction of a cancer should also be tested.

The diagnostic methods employing detectably labelled rHuAFP (or a fragment or analog thereof) of the invention may be used to detect the presence of a cancer prior to, or after the onset of, clinical symptoms associated with the cancer.

The method of the invention facilitates diagnosis of a neoplasm prior to or coincident with the onset of clinical symptoms (e.g., a palpable tumorous mass). For example, the method of the subject invention may provide a diagnosis of breast cancer prior to onset of clinical symptoms. Furthermore, the method of the invention allows the clinician to provide an accurate diagnosis of a neoplasm such as breast or prostate cancer.

Diagnostic imaging methods of the invention will be carried out using a diagnostically effective amount of a diagnostic agent of rHuAFP in a physiologically acceptable carrier. Suitable carriers and their formulation are described for example in Remington's

Pharmaceutical Sciences by E.W. Martin. The amount of the diagnostic agent to be administered varies depending upon the manner of administration, the age and body weight of the patient, and with the type of disease, extensiveness of the disease, and size of the patient suffering from the disease. Generally, however, amounts will be in the range of those used for other agents used in the diagnosis of cancer, although in certain instances lower amounts will be needed because of the increased specificity of the compound. For example, a detectably labelled rHuAFP is administered intravenously to a patient, as is described above, at a dosage that allows imaging of a neoplasm, e.g., by radioimaging using scintigraphy. Typically, a dosage is in the range of 0.1 ng - 10 g/kg body weight.

All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Cell Culture Media of the Invention

The invention furhter provides a media containing rHuAFP (or a fragment or analog thereof) for cell culture. While media of the invention generally does not require the use of serum (e.g., fetal bovine serum, calf serum, horse serum, normal mouse serum, human serum, porcine serum, rabbit serum etc.), since such rHuAFP is intended to replace or supplement the use of serum, thos skilled in the art will understand and recognize that serum can be added if desired. Media formulations are generally prepared according to methods known in the art. Accordingly, any standard medium, e.g., RMPI-1630 Medium, CMRL Medium, Dulbecco's Modified Eagle Medium (D-MEM), Fischer's Medium, Iscove's Modified Dulbecco's Medium, McCoy's Medium, Minimum Essential Medium, NCTC Medium,

and the like can be formulated with rHuAFP (or a fragment or analog thereof) at the desired effective concentration. If desired, media supplements, e.g., salt solutions (e.g., Hank's Balanced Salt Solution or Earle's Balanced Salt Solution), antibiotics, nucleic acids, amino acids, carbohydrates, and vitamins are added according to known methods. If desired, growth factors, colony-stimulating factors, cytokines and the like can also be added to media according to standard methods. For example, media of the invention can contain any of the following substances, alone or in combination, with rHuAFP (or a fragment or analog thereof): erythropoietin, granulocyte/macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), an interleukin (e.g., IL-1, IL-2, IL-3, IL-4, IL-5, etc.), insulin-growth factor (IGF), transferrin, albumin, and stem-cell growth factor (SCF). Media of the invention are useful for culturing a variety of eukaryotic cells, e.g., mammalian cells, yeast cells, amphibian cells, and insect cells. Media can also be used for culturing any tissue or organ. Such media can also be used in a variety of culture conditions and for a variety of biological applications. Examples of such culture conditions include, without limitation, bioreactors (e.g., continuous or hollow fiber bioreactors), cell-suspension cultures, semisolid cultures, liquid cultures, and long-term cell suspension cultures. Media of the invention are also useful for industrial applications, e.g., culturing hybridoma cells, genetically-engineered mammalian cells, tissues or organs.

Recombinant Human Alpha-Fetoprotein As A Cell-Proliferative Agent

Cell growth-promoting attributes of rHuAFP (or a fragment or analog thereof) is evaluated by any standard

assay f r analysis of cell proliferation in vitro and in vivo. As discussed infra, the art pr vides animal systems for in vivo testing of cell growth promoting or boosting characteristics of rHuAFP (or a fragment or analog thereof). Furthermore, a wide variety of in vitro systems are also available for testing growth-promoting or growth-boosting aspects of rHuAFP (or a fragment or analog thereof).

Any cell that proliferates in response to rHuAFP (or a fragment or analog thereof) can be identified according to standard methods known in the art. For example, proliferation of a cell (e.g., a bone marrow cell) can be monitored by culturing in a liquid media containing the test compound, either alone or in combination with other growth factors, added artificially to a serum-free or serum-based medium. Alternatively, such bone marrow cells can be cultured in a semisolid matrix of dilute agar or methylcellulose, and the test compound, alone or in combination with other growth factors, can be added artificially to a serum-free or serum-reduced medium. In the semisolid matrix the progeny of an isolated precursor cell, proliferating in response to rHuAFP or a fragment or analog thereof, remain together as a distinguishable colony. For example, a bone marrow cell may be seen to give rise to a clone of a plurality of bone marrow cells, e.g., NK cells. Such culture systems provide a facile way for assaying whether a cell responds to rHuAFP (a fragment or analog thereof) either alone or in combination with other growth factors.

If desired, identification and separation of expanded subpopulations of cells is performed according to standard methods. For example, cells may be analyzed by fluorescence-activated cell sorting (FACS). This procedure generally involves labelling cells with

antibodies coupled to a fluoresc nt dye and separating the labeled cells from the unlabelled cells in a FACS, e.g., FACScan (Becton Dickson). Thus virtually any cell can be identified and separated, e.g., by analyzing the presence of cell surface antigens (see e.g., Shah et al., J. Immunol. 140:1861, 1988). When a population of cells is obtained, it is then analyzed biochemically or, alternatively, provides a starting population for additional cell culture, allowing the action of the cells to be evaluated under defined conditions in culture.

In one working example, the effect of rHuAFP (or a fragment or analog thereof) on the growth of human bone marrow cells is examined as follows. In general, human bone marrow samples are obtained according to standard procedures after informed consent. For example, bone marrow is obtained from the iliac crest of a healthy donor and the marrow cells are diluted in phosphate-buffered saline at room temperature. Cells are then washed and cultured in an appropriate growth medium. For example, cultures can be set up by inoculating bone marrow cells in 20-30 ml of McCoy's medium containing 50 U/ml penicillin, 50 U/ml streptomycin and 2 mM L-glutamine. Cultures are incubated in the presence or absence of the test compound alone, or in combination with other growth factors, e.g., transferrin or GM-CSF. The cultures are subsequently incubated at 37°C in a humidified atmosphere containing 5% CO_2, 5% O_2, and 90% N_2 for the desired time period. Cell proliferation assays are performed according to standard methods. For example, replicate samples cultured in the presence and absence of the test compound are analyzed by pulsing the cells with 1-2 μCi of ^{3}HTdR. After an incubation period, cultures are harvested onto glass-fiber filters and the incorporated ^{3}H measured by liquid scintillation. Comparative studies between treated and control cells,

e.g., cell cultured in the presence of rHuAFP versus cells cultured in the absence of rHuAFP, are used to determine the relative efficacy of the test molecule in stimulating cell proliferation. A molecule which stimulates cell proliferation is considered useful in the invention.

To evaluate the proliferative effects of rHuAFP (or a fragment or analog thereof) e.g., the effect of the test compound on hematopoiesis in vivo, the test molecule is administered to sublethally irradiated mice (or mice treated with an immunosuppressive agent such as cyclosporine or FK-506, or a chemotherapeutic agent such as 5-fluorouracil or cyclophosphamide or any other method known in the art to deplete bone marrow) and normal mice according to standard methods, e.g., intravenously or intraperitoneally, at an appropriate dosage on a daily basis. Generally, administration of the test compound to treated mice is initiated prior to and/or after treating the animal, e.g., with sublethal radiation or immunotherapy or chemotherapy. Control animals receive a placebo, e.g., human serum albumin or diluent, similarly administered as for rHuAFP or related molecules. The effect of the test molecule on hematopoiesis is monitored by standard techniques. For example, white blood cell count in peripheral blood and spleen in both treated and control animals are analyzed. Qualitative and quantitative analyses of bone marrow, e.g., lymphocytic lineage or myeloid lineage or any other cell type, can also be determined and analyzed according to conventional methods. Comparative data between treated and control animals are used to determine the relative efficacy of the test molecule in promoting cell proliferation, e.g., stimulates bone marrow cell production, mature B lymphocyte, thymocyte, or peripheral T lymphocyte cell

producti n. A test molecule which stimulates cell proliferation is considered useful in the invention.

The following example demonstrates that unglycosylated rHuAFP stimulates the growth of bone marrow cells in vitro. This example is provided to illustrate, not limit, the invention.

EXPERIMENTAL

MATERIALS AND METHODS

Animals

Adult male and female CBA/J mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). All mice were bred and maintained in our animal facility. Animals used in this study were 12 to 20 weeks old.

Cultures

Bone marrow cells were collected by flushing the tibias and femurs of CBA/J mice with modified Dulbecco's phosphate-buffered saline (PBS) using a sterile syringe and 25-gauge needle. Homogenous single-cell suspensions were obtained by the repeated passage of cell mixtures through a Pasteur pipet. All cells were washed twice by centrifugation at 250g for 10 min in PBS and then assessed for viability by trypan blue dye exclusion. A cell viability of 95% or better was recorded in all experiments. Cells were then adjusted to the desired concentration prior to use. Bone marrow cells (250,000) were cultured in 96-well round-bottom microtiter plates (Flow Laboratories, Mississauga, Ontario, Canada). The culture medium was serum-free RPMI plus 4 mM L-glutamine, 20 mM Hepes, 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO Laboratories, Burlington, Ontario, Canada), 5 μg/ml transferrin, and 5 X 10^{-5} 2-mercaptoethanol (Eastman Chemicals Co., Rochester N.Y.). Cells were cultured in the presence or absence of rHuAFP at a concentration of 400 μg/ml, respectively. Total volume of all cultures was 0.2 ml. Cultures were maintained at 37°C in 95%

humidified air and 5% CO_2. Six hours prior to harvesting, the cultur s were pulsed with 1 μCi tritiated thymidine (NEN, sp act 77.1 Ci/mmol). Cells were then harvested on glass fiber mats (Flow Labs) with a multiple sample harvester (Skatron, Flow Labs). Water-insoluble tritiated thymidine incorporation was measured with an LKB 1215 Rackbeta II using standard liquid scintillation techniques.

RESULTS

Effects of rHuAFP on Bone Marrow Proliferation in Serum-Free Media

The effects of purified rHuAFP on cultured murine bone marrow was evaluated in serum-free medium. In this experiment, 2.5 X 10^5 viable cells from bone marrow of CBA/J mice were cultured for 72 hours in serum-free RPMI media in the presence or absence of rHuAFP at a final concentration of 400 μg/ml and transferrin at a final concentration of 5 μg/ml. Data shown in Fig. 10 indicate that bone marrow cells undergo a strong proliferative response in the presence of unglycosylated rHuAFP; with a stimulation index (SI) of 35. No such proliferation was observed when bone marrow cells were cultured in the absence of rHuAFP.

Therapy

As demonstrated above, rHuAFP is effective in promoting the proliferation of cells and accordingly is useful for therapy involving the promotion of cell proliferation, e.g., proliferation of bone marrow cells, and in treatment for the prevention of side effects of immunosuppressive therapy, radiotherapy or chemotherapy, or other therapies known to depress the immune system and suppress bone marrow production, causing myelotoxicity. Accordingly, rHuAFP (or a fragment or analog thereof) is employed to treat deficiencies in hematopoietic progenitor or stem cells, or related disorders.

Recombinant HuAFP (r a fragment r anal g ther of) may also be employed in methods for treating cancer and ther pathological states resulting in myelotoxcity, exposure to radiation or drugs, and including for example, leukopenia, bacterial and viral infections, anemia, B cell or T cell deficiencies, including immune cell or hematopoietic cell deficiency following autologous or non-autologous bone marrow transplantation. Recombinant HuAFP (or a fragment or analog thereof) may also be employed to stimulate development of megakaryocytes and natural killer cells in vitro or in vivo.

The media, compositions, and methods of the invention are also useful for treating cancers that are treated by bone marrow transplants (BMT) that involve removing bone marrow cells from the patient, maintaining these cells in an ex vivo culture while the patient is treated with radiation or chemotherapy, and then transplanting these cells back into the patient after the treatment has been completed to restore the patient's bone marrow. Accordingly, rHuAFP may be employed for BMT as a means for reconstituting bone marrow in ex vivo cell culture medium and for promoting bone marrow cell proliferation in vivo. Recombinant HuAFP (a fragment or analog thereof) is also useful for other cell therapies, e.g. cell expansion and/or gene therapy protocols, therapies requiring ex vivo cell culture. Recombinant HuAFP (a fragment or analog) is also useful in the prevention of autologous or allogenic bone marrow transplant rejection.

Therapeutic Administration

Recombinant HuAFP (or a fragment or analog thereof) can be formulated according to known methods to prepare pharmaceutically useful compositions. Recombinant human alpha-fetoprotein, e.g., rHuAFP (or a fragment or analog thereof), is preferably administered

to the patient in an amount which is effective in preventing or ameliorating the symptoms of myleotoxcity. Generally, a dosage of 0.1 ng/kg to 10 g/kg body weight is adequate. For example, treatment of human patients will be carried out using a therapeutically effective amount of rHuAFP (or a fragment or analog thereof) in a physiologically acceptable carrier. Suitable carriers and their formulation are described for example in Remington's Pharmaceutical Sciences by E.W. Martin. The amount of rHuAFP to be administered will vary depending upon the manner of administration, the age and body weight of the patient, and with the type of disease, and size of the patient predisposed to or suffering from the disease. Preferable routes of administration include, for example, oral, subcutaneous, intravenous, intrperitoneally, intramuscular, transdermal or intradermal injections which provide continuous, sustained levels of the drug in the patient. In other preferred routes of administration, rHuAFP can be given to a patient by injection or implantation of a slow release preparation, for example, in a slowly dissociating polymeric or crystalline form; this sort of sustained administration can follow an initial delivery of the drug by more conventional routes (for example, those described above). Alternatively, rHuAFP can be administered using an external or implantable infusion pump, thus allowing a precise degree of control over the rate of drug release, or through installation of rHuAFP in the nasal passages in a similar fashion to that used to promote absorption of insulin. As an alternative to nasal transmucosal absorption, rHuAFP can be delivered by aerosol deposition of the powder or solution into the lungs.

The therapeutic method(s) and compositions of the present invention may also include co-administration with

other human growth factors. Exemplary cytokines or hematopoietins for such use include, without limitation, factors such as an interleukin (e.g., IL-1), GM-CSF, G-CSF, M-CSF, tumor necrosis factor (TNF), transferrin, and erythropoietin. Growth factors like B cell growth factor, B cell differentiation factor, or eosinophil differentiation factors may also prove useful in co-administration with rHuAFP (or a fragment or analog thereof). The dosage recited above would be adjusted to compensate for such additional components in the therapeutic composition. Progress of the treated patient can be monitored by conventional methods.

Treatment is started generally with the diagnosis or suspicion of myelotoxcity and is generally repeated on a regular or daily basis to ameliorate or prevent the progression or exacerbation of the condition. Protection or prevention from the development of a myleotoxcemic condition is also achieved by administration of rHuAFP prior to the onset of the disease. If desired, the efficacy of the treatment or protection regimens is assessed with the methods of monitoring or diagnosing patients for myelotoxcity.

The method(s) of the invention can also be used to treat non-human mammals, for example, domestic pets, or livestock.

Other Embodiments

In other embodiments, the invention includes the use of rHuAFP (or fragment or analog thereof) for the prevention or treatment of acquired immunodeficiency syndrome (AIDS). To evaluate the immunosuppressive effect of rHuAFP or a fragment or analog thereof on AIDS, i.e., the compound's ability to prevent or ameliorate an autoimmune component of AIDS, test compounds are administered to an appropriate animal (e.g., a human patient), according to standard methods, e.g.,

intravenously or intrap ritoneally, at an appropriate dosage n a daily basis as is discuss d above. Generally, administration is initiated prior to the onset of AIDS and/or after the clinical appearance of AIDS. Control animals receive a placebo, e.g., human serum albumin, similarly administered as for rHuAFP or related molecules. The effect of the test compound on AIDS is monitored according to standard methods. For example, analysis of the ability of the test compound to inhibit or prevent or ameliorate the destruction of helper T cells can be monitored. Comparative studies between treated and control animals are used to determine the relative efficacy of the test compounds in preventing or ameliorating AIDS. A molecule which prevents or ameliorates (decreases or suppresses or relieves or promotes remission of) the symptoms of AIDS is considered useful in the invention.

In the invention also includes the use of a therapeutically effective amount rHuAFP (or fragment or analog thereof) for inhibiting the rejection of a transplanted organ (e.g., the heart, the liver, the lung, the pancreas, and the kidney), tissue (e.g., skin, bone marrow, dura mater, bone, implanted collagen, an implanted bioreactor), or cell (e.g., β islet cells of the pancreas, stem cells, hematopoietic cells, lymph cells, neuroendocrine or adrenal cells) in a mammal. Such transplanted organs, tissues, or cells may be derived from any source, e.g., such biological material can be allogenic, phenogenic, autologous, synthetic, artificial or genetically-engineered. For example, the method can also be used when the patient is the recipient of an allograft such a heart or kidney from another species.

In one working example, the immunosuppressive effect of rHuAFP on clinical transplantation, i.e., the

ability of rHuAFP to prevent or ameliorate transplant rejection (e.g., hyperacute rejection, acute r j ction and chronic rejection), is evaluated by administering rHuAFP to an NIH minipig according to standard methods, e.g., intravenously or intraperitoneally, at an appropriate dosage on a daily basis. Generally, administration of rHuAFP is initiated prior to the transplant, e.g., transplantation of a kidney and/or after the transplant procedure. Control animals receive a placebo, e.g., human serum albumin, similarly administered as for rHuAFP. The effect of rHuAFP on transplant rejection is monitored according to standard methods. One manifestation of the rejection process is diminished function of the transplanted organ, for example, analysis of urine output can be monitored. If desired, histological inspection (e.g., by using any standard histochemical or immunohistochemical procedure, see e.g., Ausubel et al., supra; Bancroft and Stevens, supra) of kidney tissue is performed and tissue samples obtained by biopsy are examined microscopically for evidence of transplant rejection, e.g., chronic interstitial fibrosis, vascular thrombosis, or the presence of abnormal lymphocytic infiltrates. Comparative studies between treated and control animals are used to determine the relative efficacy of rHuAFP in preventing or ameliorating transplant rejection. Recombinant HuAFP (a fragment or analog thereof) which prevents or ameliorates (decreases or suppresses or relieves or promotes remission of) the symptoms of transplant rejection is considered useful in the invention.

All publications, manufacturer's instructions, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent

applicati n was specifically and individually indicat d to be incorporated by referenc .

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Murgita, Robert A.

(ii) TITLE OF INVENTION: EXPRESSION AND PURIFICATION OF CLONED HUMAN ALPHA-FETOPROTEIN

(iii) NUMBER OF SEQUENCES: 20

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Fish & Richardson P.C.
(B) STREET: 225 Franklin Street, Suite 3100
(C) CITY: Boston
(D) STATE: MA
(E) COUNTRY: USA
(F) ZIP: 02110-2804

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

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(B) FILING DATE: 24-JAN-1996
(C) CLASSIFICATION:

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(B) FILING DATE: 24-JAN-1995
(C) CLASSIFICATION:

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(B) FILING DATE: 24-JAN-1995
(C) CLASSIFICATION:

(A) APPLICATION NUMBER: 08/377,309
(B) FILING DATE: 24-JAN-1995
(C) CLASSIFICATION:

(A) APPLICATION NUMBER: 08/377,316
(B) FILING DATE: 24-JAN-1995
(C) CLASSIFICATION:

(A) APPLICATION NUMBER: 08/505,012
(B) FILING DATE: 21-JULY-1995
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Clark, Paul T.
(B) REGISTRATION NUMBER: 30,162
(C) REFERENCE/DOCKET NUMBER: 06727/003001

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (617) 542-5070
(B) TELEFAX: (617) 542-8906
(C) TELEX: 200154

(2) INFORMATI N F R SEQ ID N :1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGTCTGCAGG ATGGGGAAAA A 21

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CATGAAATGA CTCCAGTA 18

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CATAGAAATG AATATGGA 18

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2022 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATATTGTGCT TCCACCACTG CCAATAACAA AATAACTAGC AACCATGAAG TGGGTGGAAT 60

CAATTTTTTT AATTTTCCTA CTAAATTTTA CTGAATCCAG AACACTGCAT AGAAATGAAT 120

ATGGAATAGC TTCCATATTG GATTCTTACC AATGTACTGC AGAGATAAGT TTAGCTGACC 180

TGGCTACCAT ATTTTTTGCC CAGTTTGTTC AAGAAGCCAC TTACAAGGAA GTAAGCAAAA 240

TGGTGAAAGA TGCATTGACT GCAATTGAGA AACCCACTGG AGATGAACAG TCTTCAGGGT 300

GTTTAGAAAA CCAGCTACCT GCCTTTCTGG AAGAACTTTG CCATGAGAAA GAAATTTTGG 360

AGAAGTACGG ACATTCAGAC TGCTGCAGCC AAAGTGAAGA GGGAAGACAT AACTGTTTTC 420

TTGCACACAA AAAGCCCACT GCAGCATGGA TCCCACTTTT CCAAGTTCCA GAACCTGTCA 480

CAAGCTGTGA AGCATATGAA GAAGACAGGG AGACATTCAT GAACAAATTC ATTTATGAGA 540

TAGCAAGAAG GCATCCCTTC CTGTATGCAC CTACAATTCT TCTTTCGGCT GCTGGGTATG 600

AGAAAATAAT TCCATCTTGC TGCAAAGCTG AAAATGCAGT TGAATGCTTC CAAACAAAGG 660

CAGCAACAGT TACAAAAGAA TTAAGAGAAA GCAGCTTGTT AAATCAACAT GCATGTCCAG 720

TAATGAAAAA TTTTGGGACC CGAACTTTCC AAGCCATAAC TGTTACTAAA CTGAGTCAGA 780

AGTTTACCAA AGTTAATTTT ACTGAAATCC AGAAACTAGT CCTGGATGTG GCCCATGTAC 840

ATGAGCACTG TTGCAGAGCA GATGTGCTGG ATTGTCTGCA GGATGGGGAA AAAATCATGT 900

CCTACATATG TTCTCAACAA GACACTCTGT CAAACAAAAT AACAGAATGC TGCAAACTGA 960

CCACGCTGGA ACGTGGTCAA TGTATAATTC ATGCAGAAAA TGATGAAAAA CCTGAAGGTC 1020

TATCTCCAAA TCTAAACAGG TTTTTAGGAG ATAGAGATTT TAACCAATTT TCTTCAGGGG 1080

AAAAAAATAT CTTCTTGGCA AGTTTTGTTC ATGAATATTC AAGAAGACAT CCTCAGCTTG 1140

CTGTCTCAGT AATTCTAAGA GTTGCTAAAG GATACCAGGA GTTATTGGAG AAGTGTTTCC 1200

AGACTGAAAA CCCTCTTGAA TGCCAAGATA AAGGAGAAGA AGAATTACAG AAATACATCC 1260

AGGAGAGCCA AGCATTGGCA AAGCGAAGCT GCGGCCTCTT CCAGAAACTA GGAGAATATT 1320

ACTTACAAAA TGAGTTTCTC GTTGCTTACA CAAAGAAAGC CCCCCAGCTG ACCTCGTCGG 1380

AGCTGATGGC CATCACCAGA AAAATGGCAG CCACAGCAGC CACTTGTTGC CAACTCAGTG 1440

AGGACAAACT ATTGGCCTGT GGCGAGGGAG CGGCTGACAT TATTATCGGA CACTTATGTA 1500

TCAGACATGA AATGACTCCA GTAAACCCTG GTGTTGGCCA GTGCTGCACT TCTTCATATG 1560

CCAACAGGAG GCCATGCTTC AGCAGCTTGG TGGTGGATGA AACATATGTC CCTCCTGCAT 1620

TCTCTGATGA CAAGTTCATT TTCCATAAGG ATCTGTGCCA AGCTCAGGGT GTAGCGCTGC 1680

AAAGGATGAA GCAAGAGTTT CTCATTAACC TTGTGAAGCA AAAGCCACAA ATAACAGAGG 1740

AACAACTTGA GGCTCTCATT GCAGATTTCT CAGGCCTGTT GGAGAAATGC TGCCAAGGCC 1800

AGGAACAGGA AGTCTGCTTT GCTGAAGAGG GACAAAAACT GATTTCAAAA ACTGGTGCTG 1860

CTTTGGGAGT TTAAATTACT TCAGGGGAAG AGAAGACAAA ACGAGTCTTT CATTCGGTGT 1920

GAACTTTTCT CTTTAATTTT AACTGATTTA ACACTTTTTG TGAATTAATG ATAAAGACTT 1980

TTATGTGAGA TTTCCTTATC ACAGAAATAA AATATCTCCA AA 2022

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 590 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Thr Leu His Arg Asn Glu Tyr Gly Ile Ala Ser Ile Leu Asp Ser Tyr
1 5 10 15

Gln Cys Thr Ala Glu Ile Ser Leu Ala Asp Leu Ala Thr Ile Phe Phe
20 25 30

Ala Gln Phe Val Gln Glu Ala Thr Tyr Lys Glu Val Ser Lys Met Val
35 40 45

Lys Asp Ala Leu Thr Ala Ile Glu Lys Pro Thr Gly Asp Glu Gln Ser
50 55 60

Ser Gly Cys Leu Glu Asn Gln Leu Pro Ala Phe Leu Glu Glu Leu Cys
65 70 75 80

His Glu Lys Glu Ile Leu Glu Lys Tyr Gly His Ser Asp Cys Cys Ser
85 90 95

Gln Ser Glu Glu Gly Arg His Asn Cys Phe Leu Ala His Lys Lys Pro
100 105 110

Thr Ala Ala Trp Ile Pro Leu Phe Gln Val Pro Glu Pro Val Thr Ser
115 120 125

Cys Glu Ala Tyr Glu Glu Asp Arg Glu Thr Phe Met Asn Lys Phe Ile
130 135 140

Tyr Glu Ile Ala Arg Arg His Pro Phe Leu Tyr Ala Pro Thr Ile Leu
145 150 155 160

Leu Ser Ala Ala Gly Tyr Glu Lys Ile Ile Pro Ser Cys Cys Lys Ala
165 170 175

Glu Asn Ala Val Glu Cys Phe Gln Thr Lys Ala Ala Thr Val Thr Lys
180 185 190

Glu Leu Arg Glu Ser Ser Leu Leu Asn Gln His Ala Cys Pro Val Met
195 200 205

Lys Asn Phe Gly Thr Arg Thr Phe Gln Ala Ile Thr Val Thr Lys Leu
210 215 220

Ser Gln Lys Phe Thr Lys Val Asn Phe Thr Glu Ile Gln Lys Leu Val
225 230 235 240

Leu Asp Val Ala His Val His Glu His Cys Cys Arg Ala Asp Val Leu
245 250 255

Asp Cys Leu Gln Asp Gly Glu Lys Ile Met Ser Tyr Ile Cys Ser Gln
260 265 270

Gln Asp Thr Leu Ser Asn Lys Ile Thr Glu Cys Cys Lys Leu Thr Thr
275 280 285

Leu Glu Arg Gly Gln Cys Ile Ile His Ala Glu Asn Asp Glu Lys Pro
290 295 300

Glu Gly Leu Ser Pro Asn Leu Asn Arg Phe Leu Gly Asp Arg Asp Phe
305 310 315 320

Asn Gln Phe Ser Ser Gly Glu Lys Asn Ile Phe Leu Ala Ser Phe Val
325 330 335

His Glu Tyr Ser Arg Arg His Pro Gln Leu Ala Val Ser Val Ile Leu
340 345 350

Arg Val Ala Lys Gly Tyr Gln Glu Leu Leu Glu Lys Cys Phe Gln Thr
355 360 365

Glu Asn Pro Leu Glu Cys Gln Asp Lys Gly Glu Glu Glu Leu Gln Lys
370 375 380

Tyr Ile Gln Glu Ser Gln Ala Leu Ala Lys Arg Ser Cys Gly Leu Phe
385 390 395 400

Gln Lys Leu Gly Glu Tyr Tyr Leu Gln Asn Glu Phe Leu Val Ala Tyr
405 410 415

Thr Lys Lys Ala Pro Gln Leu Thr Ser Ser Glu Leu Met Ala Ile Thr
420 425 430

Arg Lys Met Ala Ala Thr Ala Ala Thr Cys Cys Gln Leu Ser Glu Asp
435 440 445

Lys Leu Leu Ala Cys Gly Glu Gly Ala Ala Asp Ile Ile Ile Gly His
450 455 460

Leu Cys Ile Arg His Glu Met Thr Pro Val Asn Pro Gly Val Gly Gln
465 470 475 480

Cys Cys Thr Ser Ser Tyr Ala Asn Arg Arg Pro Cys Phe Ser Ser Leu
485 490 495

Val Val Asp Glu Thr Tyr Val Pro Pro Ala Phe Ser Asp Asp Lys Phe
500 505 510

Ile Phe His Lys Asp Leu Cys Gln Ala Gln Gly Val Ala Leu Gln Arg
515 520 525

Met Lys Gln Glu Phe Leu Ile Asn Leu Val Lys Gln Lys Pro Gln Ile
530 535 540

Thr Glu Glu Gln Leu Glu Ala Leu Ile Ala Asp Phe Ser Gly Leu Leu
545 550 555 560

Glu Lys Cys Cys Gln Gly Gln Glu Gln Glu Val Cys Phe Ala Glu Glu
565 570 575

Gly Gln Lys Leu Ile S r Lys Thr Gly Ala Ala Leu Gly Val
580 585 590

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 197 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Thr Leu His Arg Asn Glu Tyr Gly Ile Ala Ser Ile Leu Asp Ser Tyr
1 5 10 15

Gln Cys Thr Ala Glu Ile Ser Leu Ala Asp Leu Ala Thr Ile Phe Phe
20 25 30

Ala Gln Phe Val Gln Glu Ala Thr Tyr Lys Glu Val Ser Lys Met Val
35 40 45

Lys Asp Ala Leu Thr Ala Ile Glu Lys Pro Thr Gly Asp Glu Gln Ser
50 55 60

Ser Gly Cys Leu Glu Asn Gln Leu Pro Ala Phe Leu Glu Glu Leu Cys
65 70 75 80

His Glu Lys Glu Ile Leu Glu Lys Tyr Gly His Ser Asp Cys Cys Ser
85 90 95

Gln Ser Glu Glu Gly Arg His Asn Cys Phe Leu Ala His Lys Lys Pro
100 105 110

Thr Ala Ala Trp Ile Pro Leu Phe Gln Val Pro Glu Pro Val Thr Ser
115 120 125

Cys Glu Ala Tyr Glu Glu Asp Arg Glu Thr Phe Met Asn Lys Phe Ile
130 135 140

Tyr Glu Ile Ala Arg Arg His Pro Phe Leu Tyr Ala Pro Thr Ile Leu
145 150 155 160

Leu Ser Ala Ala Gly Tyr Glu Lys Ile Ile Pro Ser Cys Cys Lys Ala
165 170 175

Glu Asn Ala Val Glu Cys Phe Gln Thr Lys Ala Ala Thr Val Thr Lys
180 185 190

Glu Leu Arg Glu Ser
195

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 192 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not r l vant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ser Leu Leu Asn Gln His Ala Cys Pro Val Met Lys Asn Phe Gly Thr
1 5 10 15

Arg Thr Phe Gln Ala Ile Thr Val Thr Lys Leu Ser Gln Lys Phe Thr
20 25 30

Lys Val Asn Phe Thr Glu Ile Gln Lys Leu Val Leu Asp Val Ala His
35 40 45

Val His Glu His Cys Cys Arg Ala Asp Val Leu Asp Cys Leu Gln Asp
50 55 60

Gly Glu Lys Ile Met Ser Tyr Ile Cys Ser Gln Gln Asp Thr Leu Ser
65 70 75 80

Asn Lys Ile Thr Glu Cys Cys Lys Leu Thr Thr Leu Glu Arg Gly Gln
85 90 95

Cys Ile Ile His Ala Glu Asn Asp Glu Lys Pro Glu Gly Leu Ser Pro
100 105 110

Asn Leu Asn Arg Phe Leu Gly Asp Arg Asp Phe Asn Gln Phe Ser Ser
115 120 125

Gly Glu Lys Asn Ile Phe Leu Ala Ser Phe Val His Glu Tyr Ser Arg
130 135 140

Arg His Pro Gln Leu Ala Val Ser Val Ile Leu Arg Val Ala Lys Gly
145 150 155 160

Tyr Gln Glu Leu Leu Glu Lys Cys Phe Gln Thr Glu Asn Pro Leu Glu
165 170 175

Cys Gln Asp Lys Gly Glu Glu Glu Leu Gln Lys Tyr Ile Gln Glu Ser
180 185 190

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 201 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Gln Ala Leu Ala Lys Arg Ser Cys Gly Leu Phe Gln Lys Leu ly Glu
1 5 10 15

Tyr Tyr Leu Gln Asn Glu Phe Leu Val Ala Tyr Thr Lys Lys Ala Pro
20 25 30

Gln Leu Thr Ser Ser Glu Leu Met Ala Ile Thr Arg Lys Met Ala Ala
35 40 45

Thr Ala Ala Thr Cys Cys Gln Leu Ser Glu Asp Lys Leu Leu Ala Cys
50 55 60

Gly Glu Gly Ala Ala Asp Ile Ile Ile Gly His Leu Cys Ile Arg His
65 70 75 80

Glu Met Thr Pro Val Asn Pro Gly Val Gly Gln Cys Cys Thr Ser Ser
85 90 95

Tyr Ala Asn Arg Arg Pro Cys Phe Ser Ser Leu Val Val Asp Glu Thr
100 105 110

Tyr Val Pro Pro Ala Phe Ser Asp Asp Lys Phe Ile Phe His Lys Asp
115 120 125

Leu Cys Gln Ala Gln Gly Val Ala Leu Gln Arg Met Lys Gln Glu Phe
130 135 140

Leu Ile Asn Leu Val Lys Gln Lys Pro Gln Ile Thr Glu Glu Gln Leu
145 150 155 160

Glu Ala Leu Ile Ala Asp Phe Ser Gly Leu Leu Glu Lys Cys Cys Gln
165 170 175

Gly Gln Glu Gln Glu Val Cys Phe Ala Glu Glu Gly Gln Lys Leu Ile
180 185 190

Ser Lys Thr Gly Ala Ala Leu Gly Val
195 200

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 389 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Thr Leu His Arg Asn Glu Tyr Gly Ile Ala Ser Ile Leu Asp Ser Tyr
1 5 10 15

Gln Cys Thr Ala Glu Ile Ser Leu Ala Asp Leu Ala Thr Ile Phe Phe
20 25 30

Ala Gln Phe Val Gln Glu Ala Thr Tyr Lys Glu Val Ser Lys Met Val
35 40 45

Lys Asp Ala Leu Thr Ala Ile Glu Lys Pro Thr Gly Asp Glu In Ser
50 55 60

Ser Gly Cys Leu Glu Asn In Leu Pro Ala Phe Leu Glu Glu Leu Cys
65 70 75 80

His Glu Lys Glu Ile Leu Glu Lys Tyr Gly His Ser Asp Cys Cys Ser
85 90 95

Gln Ser Glu Glu Gly Arg His Asn Cys Phe Leu Ala His Lys Lys Pro
100 105 110

Thr Ala Ala Trp Ile Pro Leu Phe Gln Val Pro Glu Pro Val Thr Ser
115 120 125

Cys Glu Ala Tyr Glu Glu Asp Arg Glu Thr Phe Met Asn Lys Phe Ile
130 135 140

Tyr Glu Ile Ala Arg Arg His Pro Phe Leu Tyr Ala Pro Thr Ile Leu
145 150 155 160

Leu Ser Ala Ala Gly Tyr Glu Lys Ile Ile Pro Ser Cys Cys Lys Ala
165 170 175

Glu Asn Ala Val Glu Cys Phe Gln Thr Lys Ala Ala Thr Val Thr Lys
180 185 190

Glu Leu Arg Glu Ser Ser Leu Leu Asn Gln His Ala Cys Pro Val Met
195 200 205

Lys Asn Phe Gly Thr Arg Thr Phe Gln Ala Ile Thr Val Thr Lys Leu
210 215 220

Ser Gln Lys Phe Thr Lys Val Asn Phe Thr Glu Ile Gln Lys Leu Val
225 230 235 240

Leu Asp Val Ala His Val His Glu His Cys Cys Arg Ala Asp Val Leu
245 250 255

Asp Cys Leu Gln Asp Gly Glu Lys Ile Met Ser Tyr Ile Cys Ser Gln
260 265 270

Gln Asp Thr Leu Ser Asn Lys Ile Thr Glu Cys Cys Lys Leu Thr Thr
275 280 285

Leu Glu Arg Gly Gln Cys Ile Ile His Ala Glu Asn Asp Glu Lys Pro
290 295 300

Glu Gly Leu Ser Pro Asn Leu Asn Arg Phe Leu Gly Asp Arg Asp Phe
305 310 315 320

Asn Gln Phe Ser Ser Gly Glu Lys Asn Ile Phe Leu Ala Ser Phe Val
325 330 335

His Glu Tyr Ser Arg Arg His Pro Gln Leu Ala Val Ser Val Ile Leu
340 345 350

Arg Val Ala Lys Gly Tyr Gln Glu Leu Leu Glu Lys Cys Phe Gln Thr
355 360 365

Glu Asn Pro Leu Glu Cys Gln Asp Lys Gly Glu Glu Glu Leu Gln Lys
370 375 380

Tyr Il Gln Glu Ser
385

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 393 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ser Leu Leu Asn Gln His Ala Cys Pro Val Met Lys Asn Phe Gly Thr
1 5 10 15

Arg Thr Phe Gln Ala Ile Thr Val Thr Lys Leu Ser Gln Lys Phe Thr
20 25 30

Lys Val Asn Phe Thr Glu Ile Gln Lys Leu Val Leu Asp Val Ala His
35 40 45

Val His Glu His Cys Cys Arg Ala Asp Val Leu Asp Cys Leu Gln Asp
50 55 60

Gly Glu Lys Ile Met Ser Tyr Ile Cys Ser Gln Gln Asp Thr Leu Ser
65 70 75 80

Asn Lys Ile Thr Glu Cys Cys Lys Leu Thr Thr Leu Glu Arg Gly Gln
85 90 95

Cys Ile Ile His Ala Glu Asn Asp Glu Lys Pro Glu Gly Leu Ser Pro
100 105 110

Asn Leu Asn Arg Phe Leu Gly Asp Arg Asp Phe Asn Gln Phe Ser Ser
115 120 125

Gly Glu Lys Asn Ile Phe Leu Ala Ser Phe Val His Glu Tyr Ser Arg
130 135 140

Arg His Pro Gln Leu Ala Val Ser Val Ile Leu Arg Val Ala Lys Gly
145 150 155 160

Tyr Gln Glu Leu Leu Glu Lys Cys Phe Gln Thr Glu Asn Pro Leu Glu
165 170 175

Cys Gln Asp Lys Gly Glu Glu Glu Leu Gln Lys Tyr Ile Gln Glu Ser
180 185 190

Gln Ala Leu Ala Lys Arg Ser Cys Gly Leu Phe Gln Lys Leu Gly Glu
195 200 205

Tyr Tyr Leu Gln Asn Glu Phe Leu Val Ala Tyr Thr Lys Lys Ala Pro
210 215 220

Gln Leu Thr Ser Ser Glu Leu Met Ala Ile Thr Arg Lys Met Ala Ala
225 230 235 240

Thr Ala Ala Thr Cys Cys Gln Leu Ser Glu Asp Lys Leu Leu Ala Cys
245 250 255

Gly Glu ly Ala Ala Asp Ile Ile Il ly His Leu Cys Ile Arg His
260 265 270

Glu Met Thr Pro Val Asn Pro Gly Val Gly Gln Cys Cys Thr Ser Ser
275 280 285

Tyr Ala Asn Arg Arg Pro Cys Phe Ser Ser Leu Val Val Asp Glu Thr
290 295 300

Tyr Val Pro Pro Ala Phe Ser Asp Asp Lys Phe Ile Phe His Lys Asp
305 310 315 320

Leu Cys Gln Ala Gln Gly Val Ala Leu Gln Arg Met Lys Gln Glu Phe
325 330 335

Leu Ile Asn Leu Val Lys Gln Lys Pro Gln Ile Thr Glu Glu Gln Leu
340 345 350

Glu Ala Leu Ile Ala Asp Phe Ser Gly Leu Leu Glu Lys Cys Cys Gln
355 360 365

Gly Gln Glu Gln Glu Val Cys Phe Ala Glu Glu Gly Gln Lys Leu Ile
370 375 380

Ser Lys Thr Gly Ala Ala Leu Gly Val
385 390

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 325 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Met Ser Tyr Ile Cys Ser Gln Gln Asp Thr Leu Ser Asn Lys Ile Thr
1 5 10 15

Glu Cys Cys Lys Leu Thr Thr Leu Glu Arg Gly Gln Cys Ile Ile His
20 25 30

Ala Glu Asn Asp Glu Lys Pro Glu Gly Leu Ser Pro Asn Leu Asn Arg
35 40 45

Phe Leu Gly Asp Arg Asp Phe Asn Gln Phe Ser Ser Gly Glu Lys Asn
50 55 60

Ile Phe Leu Ala Ser Phe Val His Glu Tyr Ser Arg Arg His Pro Gln
65 70 75 80

Leu Ala Val Ser Val Ile Leu Arg Val Ala Lys Gly Tyr Gln Glu Leu
85 90 95

Leu Glu Lys Cys Phe Gln Thr Glu Asn Pro Leu Glu Cys Gln Asp Lys
100 105 110

Gly Glu Glu Glu Leu Gln Lys Tyr Ile Gln Glu Ser Gln Ala Leu Ala
115 120 125

Lys Arg Ser Cys Gly Leu Phe Gln Lys Leu Gly Glu Tyr Tyr Leu Gln
130 135 140

Asn Glu Phe Leu Val Ala Tyr Thr Lys Lys Ala Pro Gln Leu Thr Ser
145 150 155 160

Ser Glu Leu Met Ala Ile Thr Arg Lys Met Ala Ala Thr Ala Ala Thr
165 170 175

Cys Cys Gln Leu Ser Glu Asp Lys Leu Leu Ala Cys Gly Glu Gly Ala
180 185 190

Ala Asp Ile Ile Ile Gly His Leu Cys Ile Arg His Glu Met Thr Pro
195 200 205

Val Asn Pro Gly Val Gly Gln Cys Cys Thr Ser Ser Tyr Ala Asn Arg
210 215 220

Arg Pro Cys Phe Ser Ser Leu Val Val Asp Glu Thr Tyr Val Pro Pro
225 230 235 240

Ala Phe Ser Asp Asp Lys Phe Ile Phe His Lys Asp Leu Cys Gln Ala
245 250 255

Gln Gly Val Ala Leu Gln Arg Met Lys Gln Glu Phe Leu Ile Asn Leu
260 265 270

Val Lys Gln Lys Pro Gln Ile Thr Glu Glu Gln Leu Glu Ala Leu Ile
275 280 285

Ala Asp Phe Ser Gly Leu Leu Glu Lys Cys Cys Gln Gly Gln Glu Gln
290 295 300

Glu Val Cys Phe Ala Glu Glu Gly Gln Lys Leu Ile Ser Lys Thr Gly
305 310 315 320

Ala Ala Leu Gly Val
325

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 324 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Ser Tyr Ile Cys Ser Gln Gln Asp Thr Leu Ser Asn Lys Ile Thr Glu
1 5 10 15

Cys Cys Lys Leu Thr Thr Leu Glu Arg Gly Gln Cys Ile Ile His Ala
20 25 30

Glu Asn Asp lu Lys Pro Glu Gly Leu Ser Pro Asn Leu Asn Arg Phe
35 40 45

Leu Gly Asp Arg Asp Phe Asn Gln Phe Ser Ser Gly Glu Lys Asn Ile
50 55 60

Phe Leu Ala Ser Phe Val His Glu Tyr Ser Arg Arg His Pro Gln Leu
65 70 75 80

Ala Val Ser Val Ile Leu Arg Val Ala Lys Gly Tyr Gln Glu Leu Leu
85 90 95

Glu Lys Cys Phe Gln Thr Glu Asn Pro Leu Glu Cys Gln Asp Lys Gly
100 105 110

Glu Glu Glu Leu Gln Lys Tyr Ile Gln Glu Ser Gln Ala Leu Ala Lys
115 120 125

Arg Ser Cys Gly Leu Phe Gln Lys Leu Gly Glu Tyr Tyr Leu Gln Asn
130 135 140

Glu Phe Leu Val Ala Tyr Thr Lys Lys Ala Pro Gln Leu Thr Ser Ser
145 150 155 160

Glu Leu Met Ala Ile Thr Arg Lys Met Ala Ala Thr Ala Ala Thr Cys
165 170 175

Cys Gln Leu Ser Glu Asp Lys Leu Leu Ala Cys Gly Glu Gly Ala Ala
180 185 190

Asp Ile Ile Ile Gly His Leu Cys Ile Arg His Glu Met Thr Pro Val
195 200 205

Asn Pro Gly Val Gly Gln Cys Cys Thr Ser Ser Tyr Ala Asn Arg Arg
210 215 220

Pro Cys Phe Ser Ser Leu Val Val Asp Glu Thr Tyr Val Pro Pro Ala
225 230 235 240

Phe Ser Asp Asp Lys Phe Ile Phe His Lys Asp Leu Cys Gln Ala Gln
245 250 255

Gly Val Ala Leu Gln Arg Met Lys Gln Glu Phe Leu Ile Asn Leu Val
260 265 270

Lys Gln Lys Pro Gln Ile Thr Glu Glu Gln Leu Glu Ala Leu Ile Ala
275 280 285

Asp Phe Ser Gly Leu Leu Glu Lys Cys Cys Gln Gly Gln Glu Gln Glu
290 295 300

Val Cys Phe Ala Glu Glu Gly Gln Lys Leu Ile Ser Lys Thr Gly Ala
305 310 315 320

Ala Leu Gly Val

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amin acids
(B) TYPE: amino acid
(C) STRANDEDNESS: n t relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Ser Tyr Ile Cys Ser Gln Gln Asp Thr
1 5

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AAAAAAGGTA CCACACTGCA TAGAAATGAA 30

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AAAAAAGGAT CCTTAGCTTT CTCTTAATTC TTT 33

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AAAAAAATCG ATATGAGCTT GTTAAATCAA CAT 33

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AAAAAAGGAT CCTTAGCTCT CCTGGATGTA TTT 33

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AAAAAAATCG ATATGCAAGC ATTGGCAAAG CGA 33

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AAAAAAGGAT CCTTAAACTC CCAAAGCAGC ACG 33

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AAAAAAATCG ATATGTCCTA CATATGTTCT CAA 33

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GATCTAGAAT TCGGATCCGG T 21

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Asp Leu Glu Phe Met Thr Leu His Arg Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

AAAAAACTCG AGATACACTG CATAGAAATG AA 32

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucl ic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

AAAAAAGAAT TCTTAAACTC CCAAAGCAGC ACG

33

I claim:

Claims

1. Substantially pure biologically-active recombinant human alpha-fetoprotein comprising a sequence substantially identical to amino acids 1 to 389 of Fig. 1 (SEQ ID NO: 9) or a fragment thereof.

2. The pure recombinant human alpha-fetoprotein of claim 1, wherein said human alpha-fetoprotein is produced using a prokaryotic cell.

3. Substantially pure biologically-active recombinant human alpha-fetoprotein comprising a sequence substantially identical to amino acids 198 to 590 of Fig. 1 (SEQ ID NO: 10) or a fragment thereof.

4. The pure recombinant human alpha-fetoprotein of claim 3, wherein said human alpha-fetoprotein is produced using a prokaryotic cell.

5. Substantially pure biologically-active recombinant human alpha-fetoprotein comprising a sequence substantially identical to amino acids 198 to 389 of Fig. 1 (SEQ ID NO: 7) or a fragment thereof.

6. The pure recombinant human alpha-fetoprotein of claim 5, wherein said human alpha-fetoprotein is produced using a prokaryotic cell.

7. Substantially pure biologically active recombinant human alpha-fetoprotein comprising a sequence substantially identical to amino acids 390 to 590 of Fig. 1 (SEQ ID NO: 8) or a fragment thereof.

8. The pure recombinant human alpha-fetoprotein of claim 7, wherein said human alpha-fetopr t in is produced using a prokaryotic cell.

9. Substantially pure biologically active recombinant human alpha-fetoprotein comprising a sequence substantially identical to amino acids 267 to 590 of Fig. 1 (SEQ ID NO: 11) or a fragment thereof.

10. The pure recombinant human alpha-fetoprotein of claim 9, wherein said human alpha-fetoprotein is produced using a prokaryotic cell.

11. A therapeutic composition comprising the substantially pure human recombinant alpha-fetoprotein of claims 1, 3, 5, 7, and 9.

12. A method for using an insect cell for producing biologically active recombinant human alpha-fetoprotein or a fragment or analog thereof comprising

a) providing a transformed insect cell comprising a recombinant DNA molecule encoding said human alpha-fetoprotein or fragment or analog thereof operably linked to an expression control element which directs the expression of said human alpha-fetoprotein or fragment or analog thereof;

b) culturing said transformed cell; and

c) recovering said biologically active human alpha-fetoprotein or fragment or analog thereof.

13. The method of claim 12, wherein said insect cell is *Spodoptera frugiperda*.

14. Substantially pur human alpha-fetoprotein or fragm nt or analog ther of produced by the method of claim 12.

15. A therapeutic composition comprising the substantially pure human alpha-fetoprotein or fragment or analog thereof of claim 14.

16. A method of inhibiting autoreactive immune cell proliferation in a mammal, said method comprising administering to said mammal a therapeutically effective amount of recombinant human alpha-fetoprotein or an immune cell anti-proliferative fragment or analog thereof.

17. The method of claim 16, wherein said immune cells include T cells.

18. The method of claim 16, wherein said immune cells include B cells.

19. The method of claim 16, wherein said mammal is a human patient.

20. The method of claim 16, wherein said recombinant alpha-fetoprotein is produced in a prokaryotic cell and is unglycosylated.

21. The method of claim 20, wherein said prokaryotic cell is E. coli.

22. A method of treating an autoimmune disease in a mammal, said method comprising administering to said mammal a therapeutically effective amount of recombinant

human alpha-fetoprotein or an immune cell anti-proliferative fragment or analog thereof.

23. The method of claim 22, wherein said autoimmune disease is multiple sclerosis.

24. The method of claim 22, wherein said autoimmune disease is rheumatoid arthritis.

25. The method of claim 22, wherein said autoimmune disease is myasthenia gravis.

26. The method of claim 22, wherein said autoimmune disease is insulin-dependent diabetes mellitus.

27. The method of claim 22 wherein said autoimmune disease is systemic lupus erythematosus.

28. The method of claim 22, wherein said recombinant alpha-fetoprotein is produced in a prokaryotic cell and is unglycosylated.

29. The method of claim 28, wherein said prokaryotic cell is E. coli.

30. The method of claim 16, further comprising administering to said mammal an immunosuppressive agent in an effective dose which is lower than the standard dose when said immunosuppressive agent is used by itself.

31. The method of claim 16, further comprising administering to said mammal a tolerizing agent.

32. The method of claim 30 or claim 31, wherein said recombinant human alpha-fetoprotein is produced in a prokaryotic cell and is unglycosylated.

33. The method of claim 32, wherein said prokaryotic cell is *E. coli*.

34. The method of claim 30, wherein said immunosuppressive agent is cyclosporine.

35. The method of claim 30, wherein said immunosuppressive agent is a steroid, azathioprine, FK-506, or 15-deoxyspergualin.

36. A method of inhibiting a neoplasm in a mammal, said method comprising administering to said mammal a therapeutically effective amount of recombinant human alpha-fetoprotein or an anti-neoplasm fragment or analog thereof.

37. The method of claim 36, wherein said mammal is a human patient.

38. The method of claim 36, wherein said neoplasm is a malignant tumor.

39. The method claim 38, wherein said malignant tumor is a breast tumor.

40. The method of claim 38, wherein said malignant tumor is a prostate tumor.

41. The method of claim 36, wherein said recombinant human alpha-fetoprotein is produced in a prokaryotic cell and is unglycosylated.

42. The method of claim 41, wherein said prokaryotic cell is *E. coli*.

43. The method of claim 36, wherein cells of said neoplasm express a receptor which is recognized by said recombinant human alpha-fetoprotein.

44. The method of claim 36, wherein said neoplasm is a carcinoma.

45. The method of claim 36, wherein said neoplasm is an adenocarcinoma.

46. The method of claim 36, wherein said neoplasm is a sarcoma.

47. The method of claim 36, wherein said neoplasm proliferates in response to estrogen.

48. The method of claim 36, wherein said administering inhibits proliferation of cells of said neoplasm in said mammal.

49. The method of claim 36, wherein said administering kills cells of said neoplasm in said mammal.

50. The method of claim 36, further comprising administering to said mammal a chemotherapeutic agent in an effective dose which is lower than the standard dose when said chemotherapeutic agent is used by itself.

51. A method of protecting a mammal from developing a neoplasm, comprising administering to said

mammal a therapeutically effective amount of recombinant human alpha-fetoprotein.

52. The method of claim 51, wherein said recombinant human alpha-fetoprotein is produced in a prokaryotic cell and is unglycosylated.

53. The method of claim 52, wherein said prokaryotic cell is E. coli.

54. A hybrid cytotoxin comprising recombinant human alpha-fetoprotein linked to a cytotoxic agent.

55. The hybrid cytotoxin of claim 54, wherein said cytotoxic agent is a protein.

56. The hybrid cytotoxin of claim 54, wherein said cytotoxic agent is chemically conjugated to said recombinant human alpha-fetoprotein.

57. The hybrid cytotoxin of claim 54, wherein said cytotoxin is linked by a peptide bond to said recombinant human alpha-fetoprotein, and said hybrid toxin is produced by expression of a genetically engineered hybrid DNA molecule.

58. A detectably-labelled recombinant human alpha-fetoprotein or a detectably-labelled fragment or analog thereof capable of binding to a human neoplastic cell.

59. The molecule of claim 58 labelled with a radionuclide.

60. The molecule of claim 59, wh rein said radionuclide is technetium-99m.

61. The molecule of claim 58, wherein said recombinant human alpha-fetoprotein is produced in a prokaryotic cell and is unglycosylated.

62. The molecule of claim 61, wherein said prokaryotic cell is E. coli.

63. A method of imaging a neoplastic cell-containing region in a human patient in vivo, said method comprising:

(a) providing a detectably-labelled molecule of claim 58;

(b) administering said molecule to said patient;

(c) allowing said labelled molecule to bind and allowing unbound molecule to be cleared from said region containing said region; and

(d) obtaining an image of said neoplastic cell-containing region.

64. The method of claim 63, wherein said region is the breast.

65. The method of claim 63, wherein said region is the prostate.

66. The method of claim 63, wherein said region is bone marrow.

67. The method of claim 63, wherein said region is the liver.

68. Th method of claim 63, wherein said image is obtain d using scintigraphy.

69. A method for diagnosing a neoplasm in a biological sample, said method comprising:

(a) contacting said biological sample with the detectably-labelled molecule of claim 58; and

(b) detecting said label bound to said sample, wherein the detection of label above background levels is indicative that said patient has a neoplasm.

70. The method of claim 69, wherein said biological sample comprises cells fixed and sectioned prior to said contacting step, and said label bound to said sample is bound to areas corresponding to the cell membrane of said cells.

71. The method of claim 69, wherein said biological sample is from the breast of a human patient.

72. The method of claim 69, wherein said biological sample is from the prostate of a human patient.

73. A method of detecting a neoplasm a mammal in vivo, comprising:

(a) administering a diagnostically effective amount of the detectably-labelled molecule of claim 58; and

(c) detecting the presence of said detectable label bound to a tissue of said mammal, wherein an amount of label above background levels is indicative of the presence of said neoplasm in said mammal.

74. The method of claim 73, wherein said mammal is a human patient suspected of having a breast cancer, and said tissue is breast tissue.

75. The method of claim 73, wherein said mammal is a human patient suspected of having a prostate cancer, and said tissue is prostate tissue.

76. The method of claim 73, wherein said detectable label is a radionuclide and said detection step is accomplished by radioimaging.

77. The method of claim 76, wherein said radionuclide is technetium-99m and said radioimaging is scintigraphy.

78. A kit comprising

(a) a first reagent comprising recombinant human alpha-fetoprotein or a fragment or analog thereof; and

(b) a second reagent comprising a detectable label.

79. The kit of claim 78, wherein said detectable label is a radionuclide.

80. The kit of claim 79, wherein said radionuclide is technetium-99m.

81. The kit of claim 79, wherein said radionuclide includes iodine or indium.

82. The kit of claim 78, wherein said kit further includes a third reagent for linking said detectable label to said recombinant human alpha-fetoprotein or a fragment or analog thereof.

83. The kit of claim 78, wherein said detectable label includ s an enzyme, a flurophore, or an antibody.

84. The kit of claim 78, wherein said kit further includes a fourth reagent for detecting said detectable label linked to said recombinant human alpha-fetoprotein or a fragment or analog thereof.

85. The kit of claim 78, wherein said recombinant human alpha-fetoprotein is produced in a prokaryotic cell and is unglycosylated.

86. The kit of claim 78, wherein said prokaryotic cell is E. coli.

87. A cell culture medium comprising recombinant human alpha-fetoprotein or a cell-stimulating fragment or analog thereof.

88. The medium of claim 87, wherein said recombinant human alpha-fetoprotein is produced in a prokaryotic cell and is unglycosylated.

89. The media of claim 88, wherein said prokaryotic cell is E. coli.

90. A method of cell culture, said method comprising (a) providing the culture medium of claim 87; (b) providing a cell; and (c) growing said cell in said medium, wherein said cell proliferates, and is maintained.

91. The method of claim 90, wherein said cell is a mammalian cell.

92. Th method of claim 91, wherein said cell is a bon marrow cell.

93. The method of claim 92, wherein said bone marrow cell is a T cells.

94. The method of claim 92, wherein said bone marrow cell is a natural killer cell.

95. The method of claim 92, wherein said bone marrow cell is a lymphocyte.

96. The method of claim 91, wherein said cell is a hybridoma.

97. The method of claim 90, wherein said method involves ex yivo cell culture.

98. A method for inhibiting myelotoxcity in a mammal comprising administering to said mammal a therapeutically effective amount of recombinant human alpha-fetoprotein or a myelotoxic-inhibiting analog or fragment thereof.

99. The method of claim 98, wherein said mammal is a human patient.

100. The method of claim 99, wherein said recombinant human alpha-fetoprotein is produced in a prokaryotic cell and is unglycosylated.

101. The method of claim 100, wherein said prokaryotic cell is E. coli.

102. A method of inhibiting suppression of bone marrow cell proliferation in a mammal, said method comprising administering to said mammal an effective amount of recombinant alpha-fetoprotein or an anti-suppressive fragment or analog thereof.

103. The method of claim 102, wherein said recombinant human alpha-fetoprotein is produced in a prokaryotic cell and is unglycosylated.

104. The method of claim 103, wherein said prokaryotic cell is E. coli.

105. A method of promoting bone marrow cell proliferation in a mammal, said method comprising administering to said mammal an effective amount of recombinant human alpha-fetoprotein or a cell-stimulating fragment or analog thereof.

106. The method of claim 105, wherein said recombinant human alpha-fetoprotein is produced in a prokaryotic cell and is unglycosylated.

107. The method of claim 106, wherein said prokaryotic cell is E. coli.

108. A method of preventing bone marrow cell transplantation rejection in a mammal, said method comprising administering to said mammal an effective amount of recombinant human alpha-fetoprotein or an anti-rejection fragment or analog thereof.

109. The method of claim 108, wherein said recombinant human alpha-fetoprotein is produced in a prokaryotic cell and is unglycosylated.

110. The method of claim 109, wherein said prokaryotic cell is E. coli.

Figure 1

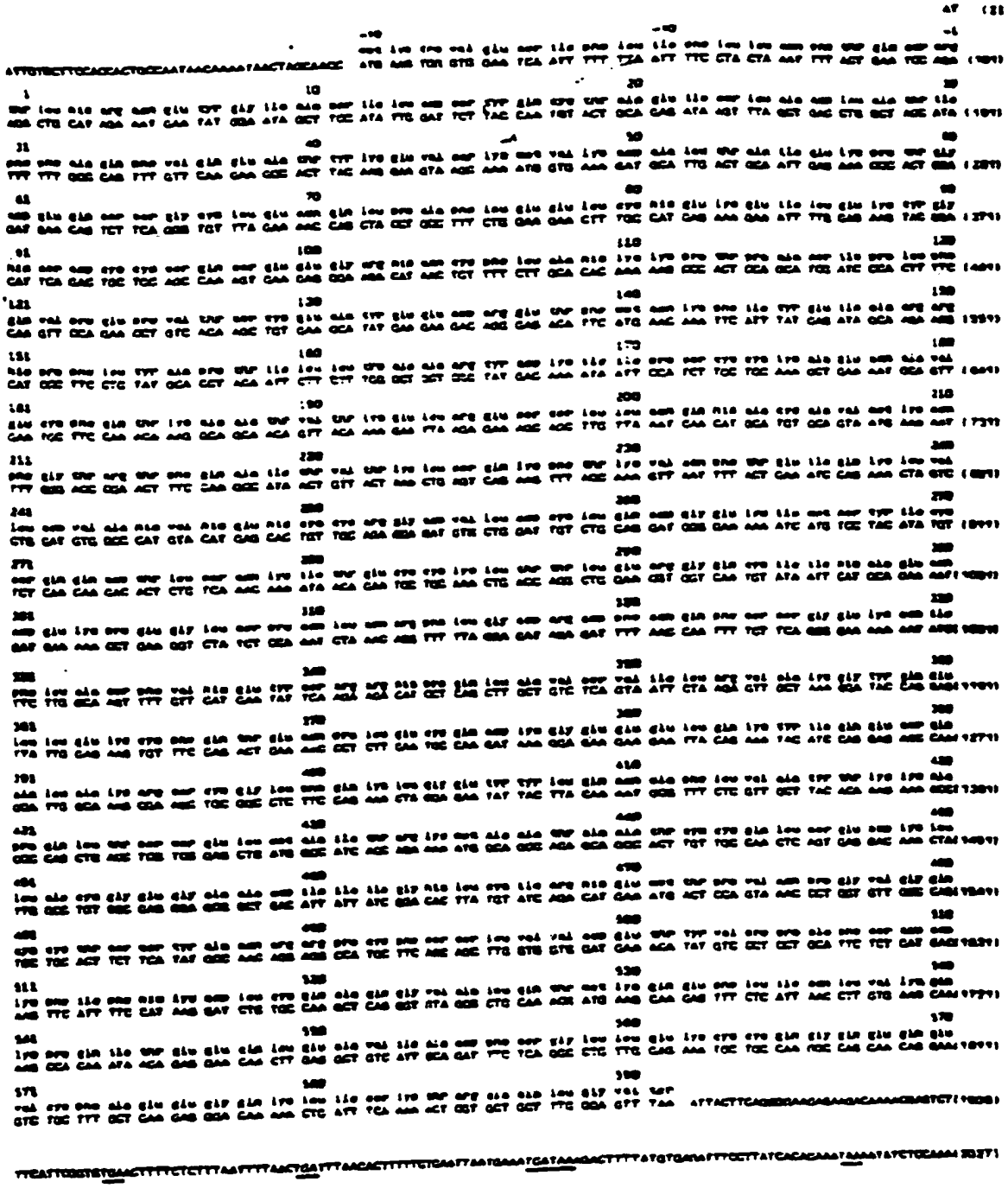


Figure 2

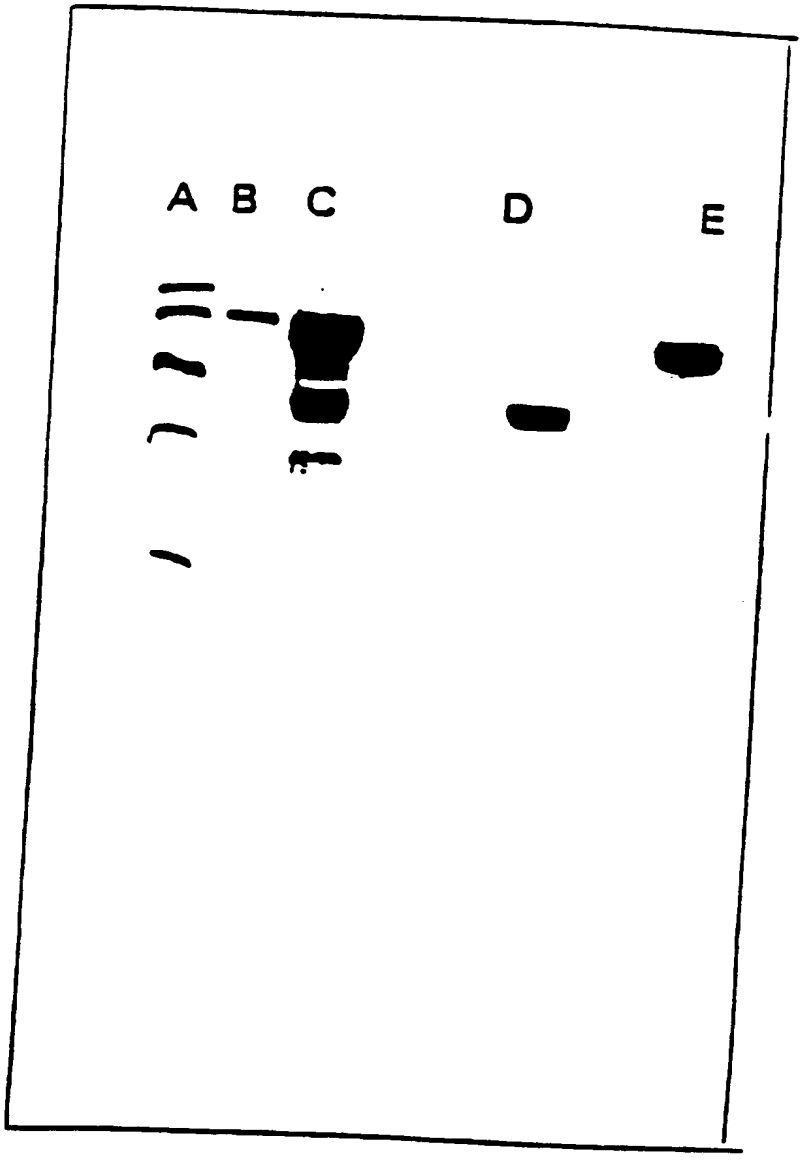


Figure 3

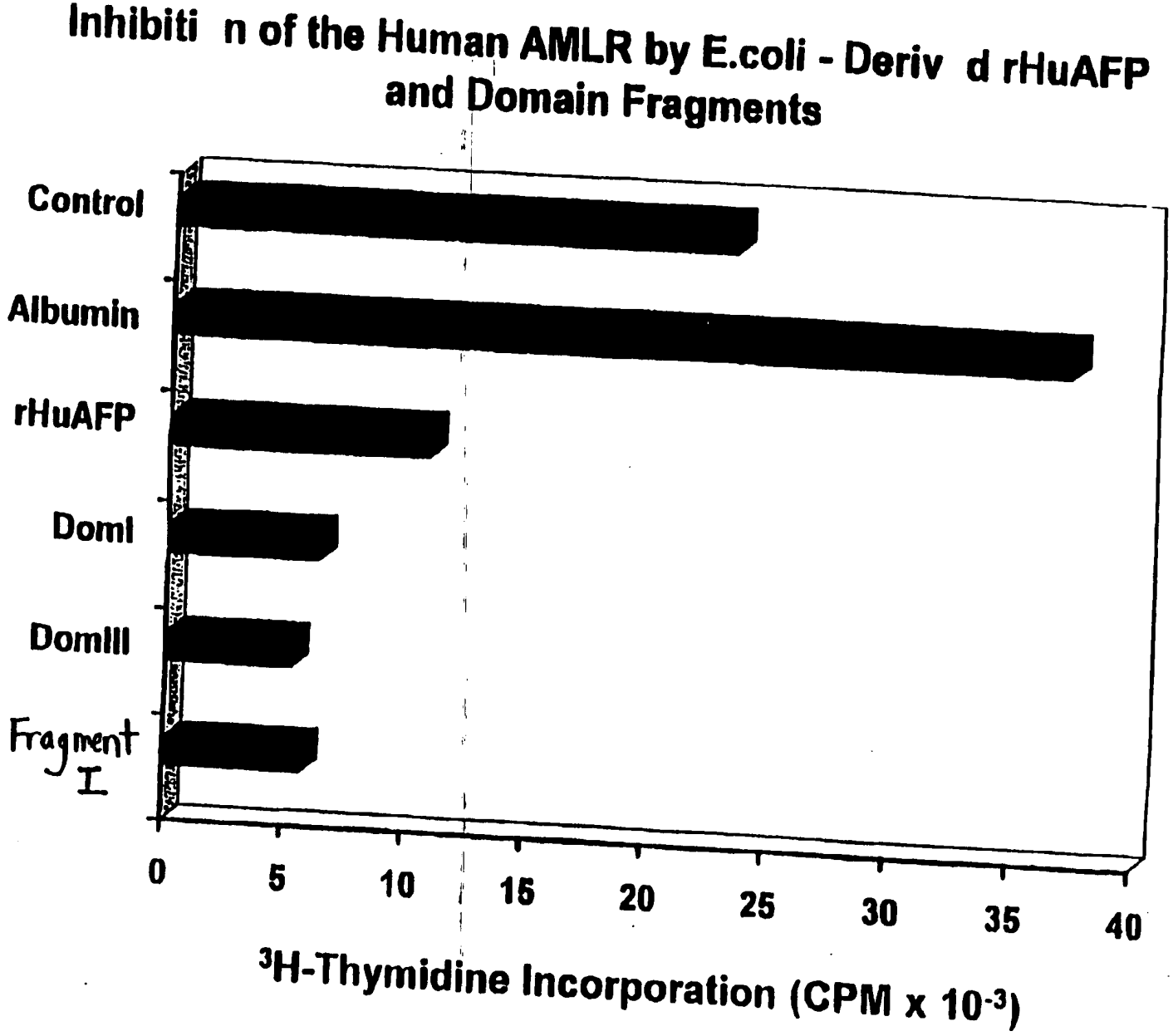

Inhibiti n of the Human AMLR by E.coli - Deriv d rHuAFP and Domain Fragments
Control
Albumin
rHuAFP
DomI
DomIII
Fragment I
0
5
10
15
20
25
30
35
40
3H-Thymidine Incorporation (CPM x 10-3)

Figure 4

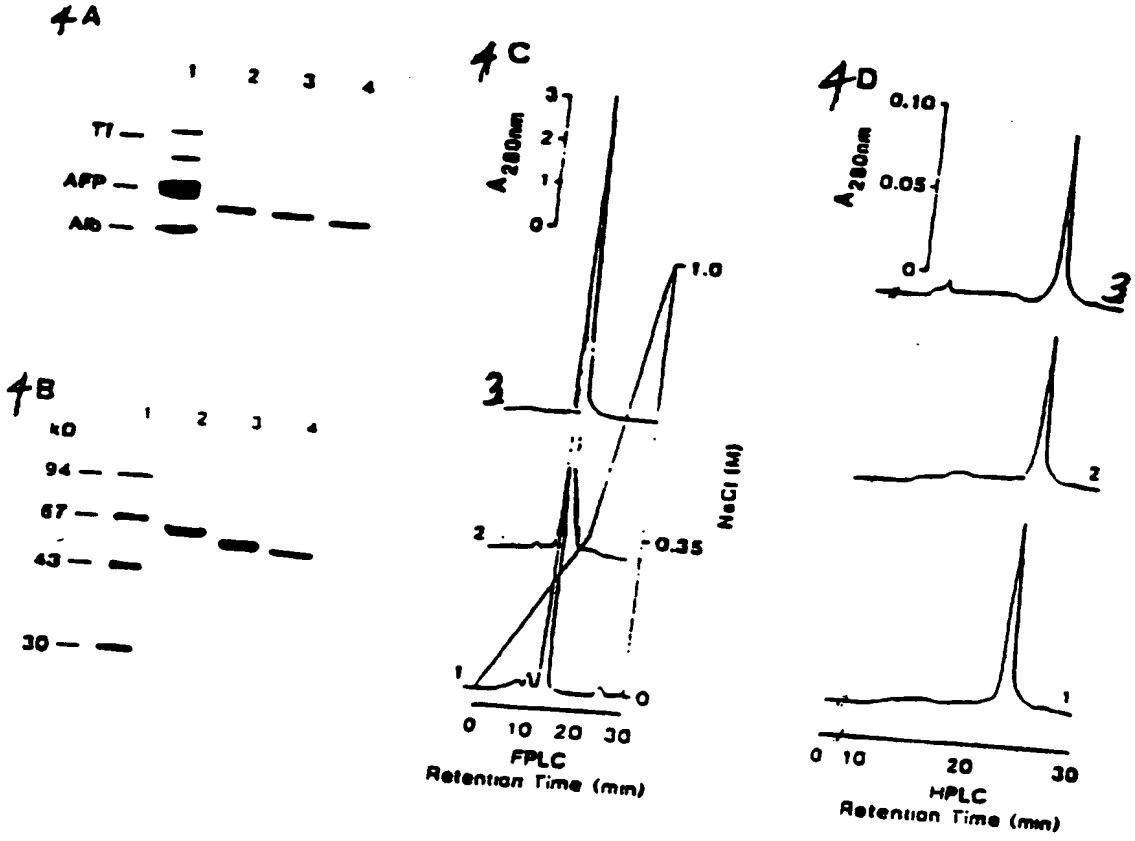

4A
1 2 3 4
Tf
AFP
Alb
4B
kD
1 2 3 4
94
67
43
30
4C
A280nm
3
2
1
0
1.0
0.35
NaCl (M)
0 10 20 30
FPLC
Retention Time (mn)
4D
A280nm
0.10
0.05
0
0 10 20 30
HPLC
Retention Time (mn)

Figure 5

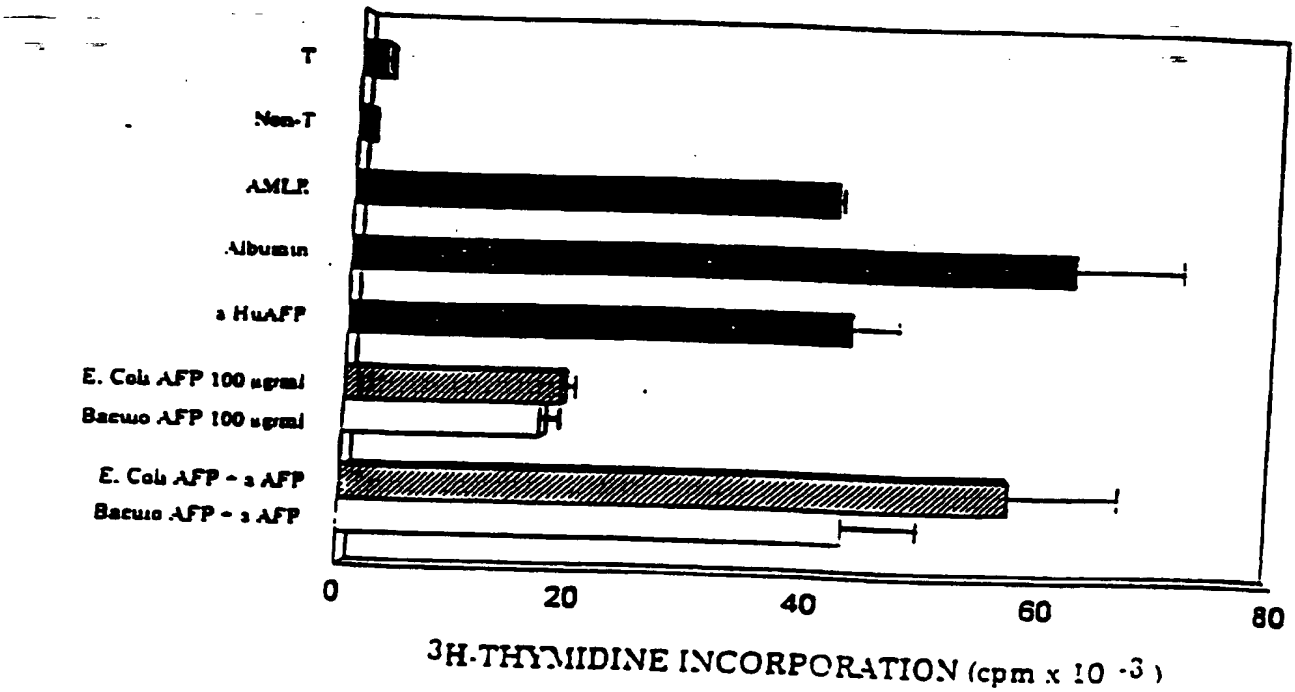
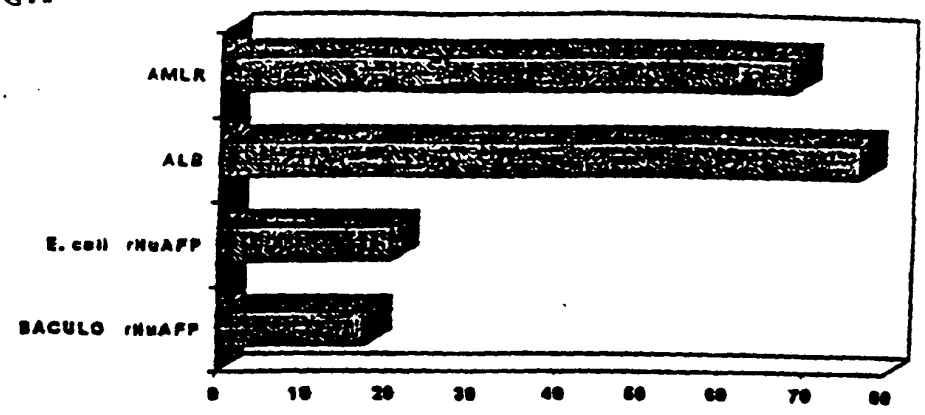

T
Non-T
AMLR
Albumin
a HuAFP
E. Coli AFP 100 ug/ml
Bacuo AFP 100 ug/ml
E. Coli AFP + a AFP
Bacuo AFP + a AFP
0
20
40
60
80
3H-THYMIDINE INCORPORATION (cpm x 10 -3)

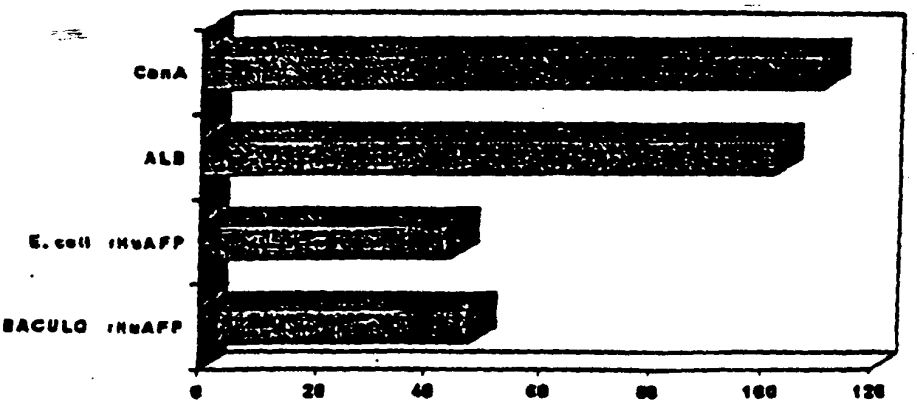
Figure 6

6A



^{3}H-THYMIDINE INCORPORATION (CPM X 10^{-3})

6B



^{3}H-THYMIDINE INCORPORATION (CPM X 10^{-3})

Figure 7

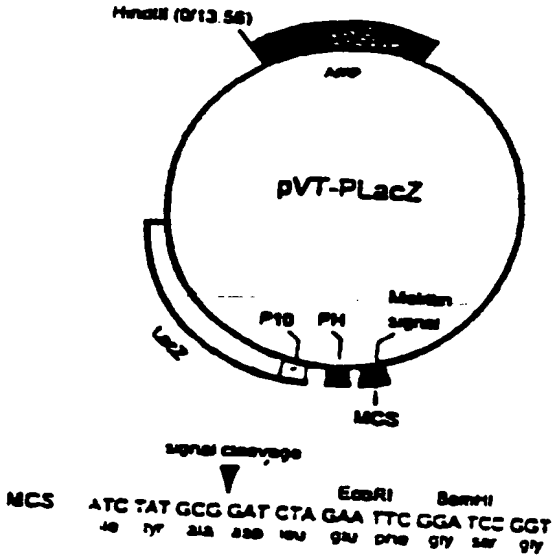

HindIII (0/13.56)
Amp
pVT-PLacZ
P10
PH
Melittin signal
MCS
Signal cleavage
MCS
ATC TAT GCG GAT CTA GAA TTC GGA TCC GGT
EcoRI
BamHI

Figure 8

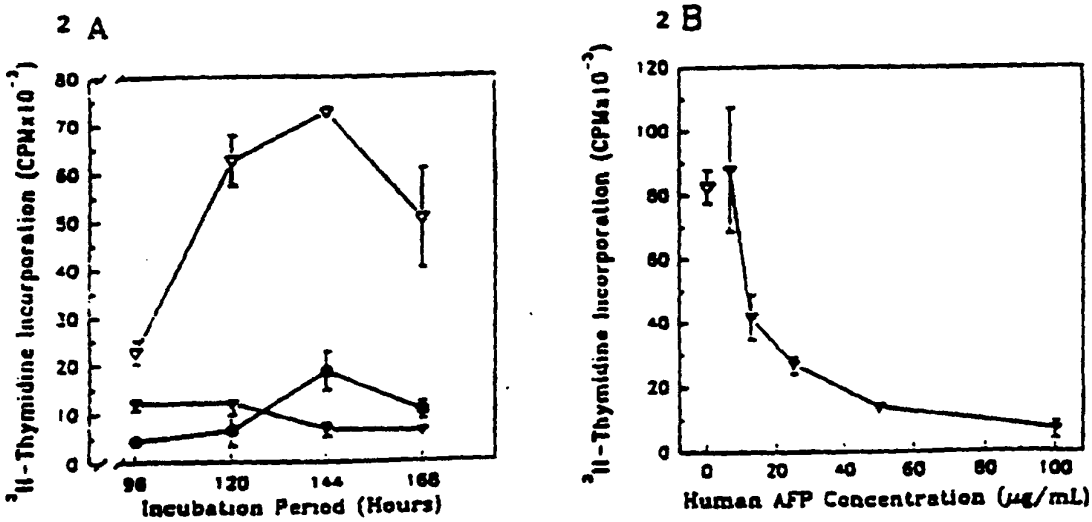

2 A
^{3}H-Thymidine Incorporation (CPMx10^{-3})
80
70
60
50
40
30
20
10
0
96
120
144
168
Incubation Period (Hours)
2 B
^{3}H-Thymidine Incorporation (CPMx10^{-3})
120
100
80
60
40
20
0
0
20
40
60
80
100
Human AFP Concentration (μg/mL)

Figure 9

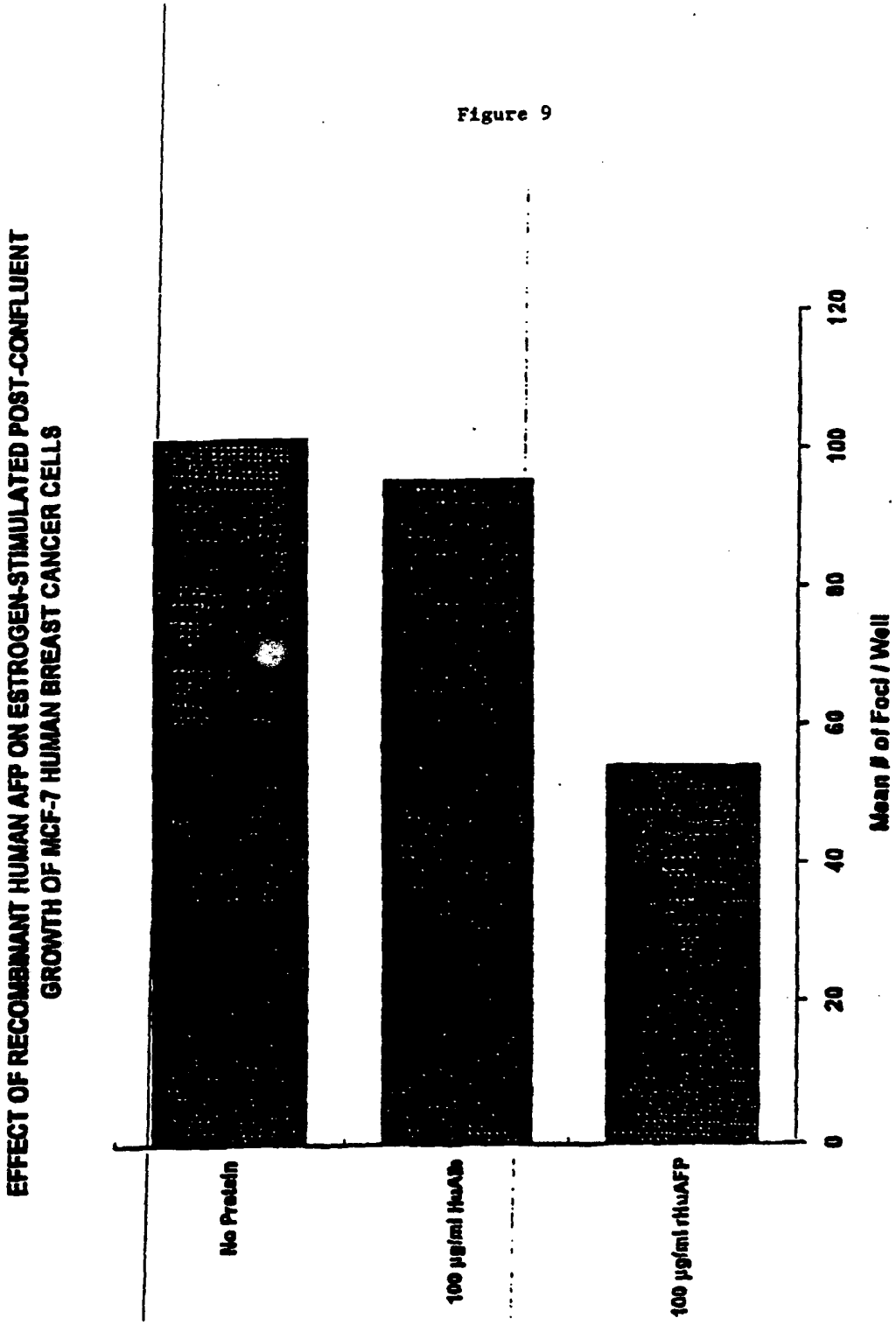
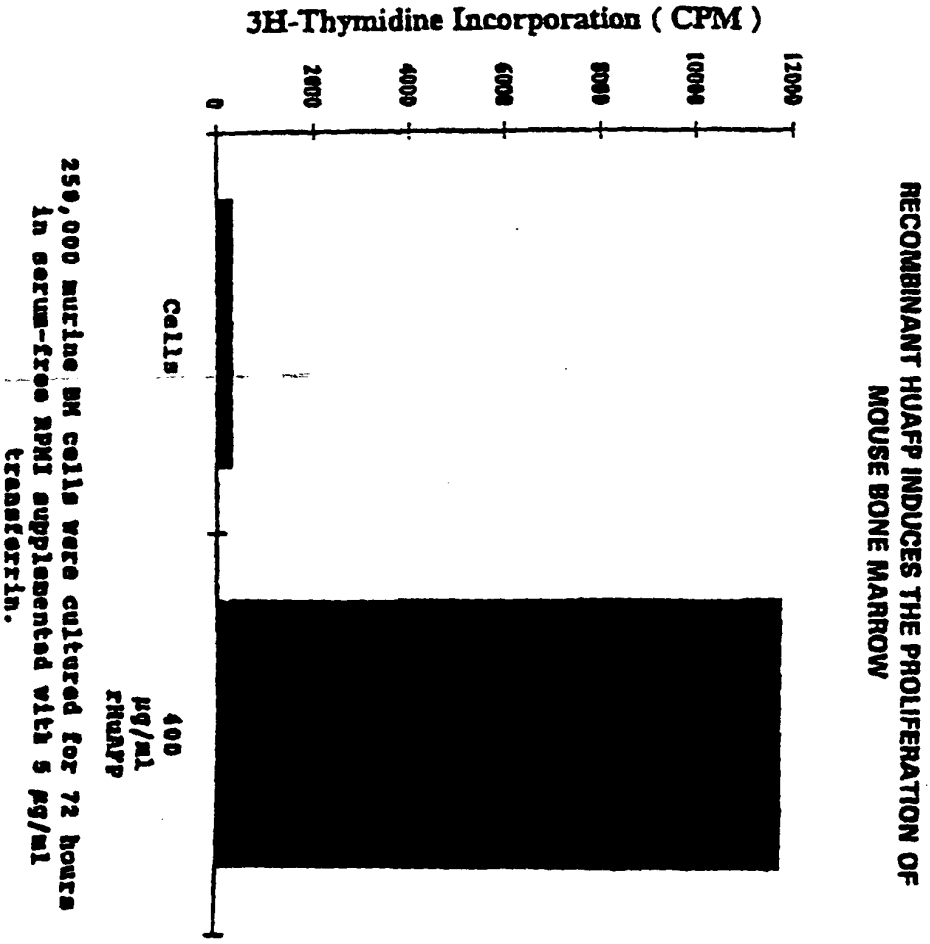

EFFECT OF RECOMBINANT HUMAN AFP ON ESTROGEN-STIMULATED POST-CONFLUENT GROWTH OF MCF-7 HUMAN BREAST CANCER CELLS
Mean # of Foci / Well
0
20
40
60
80
100
120
No Protein
100 µg/ml HuAb
100 µg/ml rHuAFP

Figure 10


RECOMBINANT HUAFP INDUCES THE PROLIFERATION OF MOUSE BONE MARROW
3H-Thymidine Incorporation (CPM)
0
2000
4000
6000
8000
10000
12000
Cells
400 µg/ml rHuAFP
250,000 murine BM cells were cultured for 72 hours in serum-free RPMI supplemented with 5 µg/ml transferrin.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/00996

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/17; C07K 14/00; C12P 21/02
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 69.6, 70.1, 70.3, 71.2; 514/2, 825, 866, 885, 903; 530/350, 380; 930/10

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 94/10199 A1 (MCGILL UNIVERSITY) 11 May 1994, see entire document.	1-10, 12-15 --- 16-35
Y	US 5,342,625 A (HAUER ET AL) 30 August 1994, column 1, line 52 to column 2, line 10.	30, 32-34
Y	US 5,011,844 A (T. FEHR) 30 April 1991, column 8, lines 17-30.	30, 32, 33, 35
Y	BENJAMIN et al., "Induction of tolerance by monoclonal antibody therapy". Letters to Nature. 03 April 1 35, Vol. 320, pages 449-451, especially pages 449 and 451.	31

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 10 MAY 1996	Date of mailing of the international search report 20 MAY 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer STEPHEN GUCKER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/00996

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MURGITA et al., "Effects of human alpha-foetoprotein on human B and T lymphocyte proliferation in vitro". Clin. Exp. Immunol. 1978, Vol. 33, pages 347-356, especially page 354.	16-35
Y	HOSKIN et al., "In Vitro Activation of Bone Marrow-Derived T- and Non-T-Cell Subsets by alpha-fetoprotein". Cellular Immunology, 1985, Vol. 96, pages 163-174, especially page 173.	16-35

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/00996

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/69.1, 69.6, 70.1, 70.3, 71.2; 514/2, 825, 866, 885, 903; 530/350, 380; 930/10

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, SCISEARCH, EMBASE, BIOSIS, CAPLUS, BIOTECHDS, DISSABS, CONFSCI, LIFESCI
search terms: fetoprotein, proliferat####, autoimmune, sclerosis, arthritis, myasthenia, diabetes, lupus, cyclosporine, FK-506

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-10, 12-35, drawn to alpha-fetoprotein, therapeutic compositions containing alpha-fetoprotein, method of making alpha-fetoprotein recombinantly, and a method to treat autoimmune diseases by using alpha-fetoprotein.

Group II, claims 36-53, drawn to a method of treating neoplasms by using alpha-fetoprotein.

Group III, claims 54-57, drawn to a hybrid cytotoxin wherein alpha-fetoprotein is part of a targeted fusion protein.

Group IV, claims 58-86, drawn to methods of imaging, detecting, and diagnosing neoplasms by using detectably-labelled alpha-fetoprotein and the detectably-labelled alpha-fetoprotein itself.

Group V, claims 87-97, drawn to a method of cell culture using alpha-fetoprotein to stimulate proliferation and the cell culture medium itself.

Group VI, claims 98-110, drawn to methods of treating bone marrow cell disorders by using alpha-fetoprotein.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Groups I-VI do not share a special technical feature because alpha-fetoprotein, which is the technical feature and inventive link shared by Groups I-VI, does not define a contribution over the prior art as defined in PCT Rule 13.2.

Group I is drawn to the special technical feature of a method of treating autoimmune disease by using alpha-fetoprotein.

Group II is drawn to the special technical feature of a method of treating neoplasms by using alpha-fetoprotein, and does not share the special technical feature of a method of treating autoimmune disease by using alpha-fetoprotein of Group I.

Group III is drawn to the special technical feature of a hybrid cytotoxin wherein alpha-fetoprotein is part of a targeted fusion protein, and does not share the special technical feature of a method of treating autoimmune disease by using alpha-fetoprotein of Group I.

Group IV is drawn to the special technical feature of methods ofimaging, detecting, and diagnosing neoplasms byusingdetectably-labelled alpha-fetoprotein and the detectably-labelled alpha-fetoprotein itself, and does not share the special technical feature of a method of treating autoimmune disease by using alpha-fetoprotein of Group I.

Group V is drawn to the special technical feature of a method of cell culture using alpha-fetoprotein to stimulate proliferation and the cell culture medium itself, and does not share the special technical feature of a method of treating autoimmune disease by using alpha-fetoprotein of Group I.

Group VI is drawn to the special technical feature of methods of treating bone marrow cell disorders by using alpha-fetoprotein, and does not share the special technical feature of a method of treating autoimmune disease by using alpha-fetoprotein of Group I.

International application No.
PCT/US96/00996

Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.